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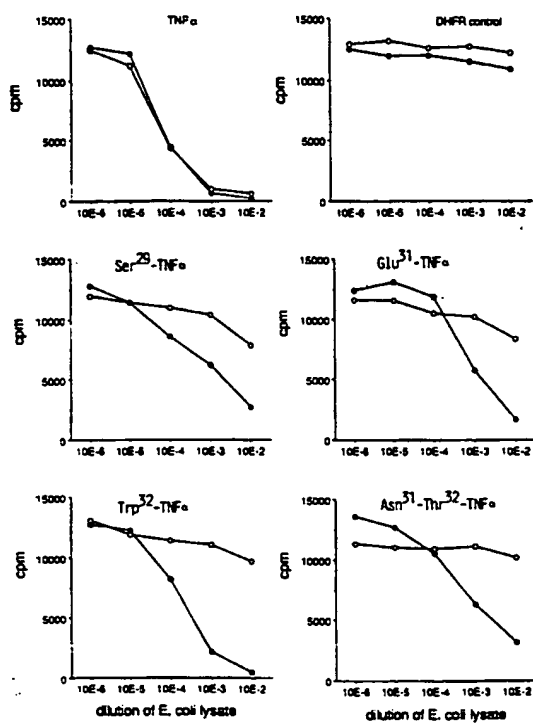
(54) **TNF-Muteins.**

(57) It is an object of the present invention to provide a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the hu-

man p55-Tumor-Necrosis-Factor-Receptor, DNA sequences coding for such muteins, vectors comprising such DNA sequences, host cells transformed with such vectors and a process for the production of such muteins employing such transformed host cells and pharmaceutical compositions containing such muteins and their use for the treatment of illnesses, e.g. cancer.

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Fig. 6





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which under Rule 45 of the European Patent Convention  
shall be considered, for the purposes of subsequent  
proceedings, as the European search report

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	JOURNAL OF BIOCHEMISTRY, vol. 101, 1987, pages 919-925, Japanese Biochemical Society, Tokyo, JP; M. TSUJIMOTO et al.: "Comparative studies of the biological activities of human tumor necrosis factor and its derivatives" * Whole article, especially figure 1 *	1-3,15-16,18-25	C 12 N 15/28 C 12 P 21/02 C 07 K 13/00 C 12 N 1/21 // A 61 K 37/02 (C 12 N 1/21 C 12 R 1:19 )
D,X	WO-A-8 806 625 (CETUS CORP.) * Page 17, lines 16-33; page 14, lines 15-31; claims *	1-3,8,11-12,15-25	
D,X	EP-A-0 168 214 (GENENTECH, INC.) * Page 63, line 16; page 65, line 9; claims *	1-4,8-25	
	--- -/-		
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 N C 07 K C 12 P
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely : Claims searched incompletely : Claims not searched : Reason for the limitation of the search:</p> <p>Remark: Although claim 25 is directed to a method of treatment of the human body (Art. 52 (4) EPC) the search has been carried out and based on the alleged effects of the compound.</p>			
Place of search THE HAGUE		Date of completion of the search 28-07-1992	Examiner LE CORNEC N.D.R.
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>A : technological background O : non-written disclosure P : intermediate document</p> <p>A : member of the same patent family, corresponding document</p>	



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Application Number

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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. CL.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	PROTEIN ENGINEERING INTERNATIONAL CONFERENCE CENTER, Kobe, 20th - 25th August 1989, pages 375-376, Eynsham, Oxford, GB; T. MASEGI et al.: "Fundamental studies on novel recombinant tumor necrosis factor mutants" * Whole document *	1-3, 8, 17-21	TECHNICAL FIELDS SEARCHED (Int. CL.5)
D,X	PROTEIN ENGINEERING, vol. 3, no. 8, August 1990, pages 713-719, Eynsham, Oxford, GB; J. YAMAGISHI et al.: "Mutational analysis of structure-activity relationships in human tumor necrosis factor-alpha" * Whole article *	1	
X	PATENT ABSTRACTS OF JAPAN, vol. 13, no. 119 (C-579)[3467], 23rd March 1989; & JP-A-63 291 590 (TEIJIN LTD) 29-11-1988 * Whole abstract *	1-3, 8, 18-25	
P,X	THE EMBO JOURNAL, vol. 10, no. 4, April 1991, pages 827-836, Eynsham, Oxford, GB; X. VAN OSTADE et al.: "Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis" * Whole article *	1-5, 8-9, 18-25	

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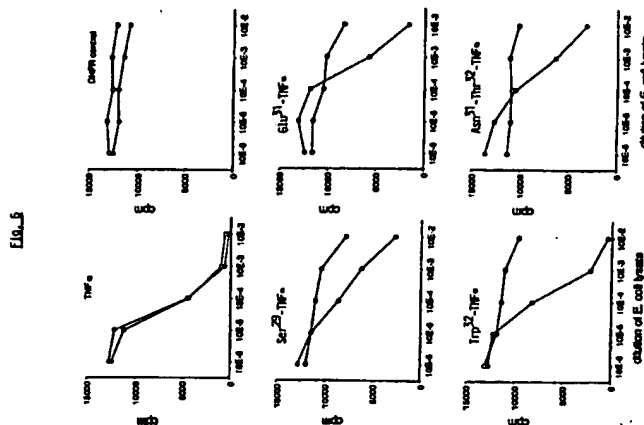
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(54) **TNF-Mutains.**

(57) It is an object of the present invention to provide a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor, DNA sequences coding for such muteins, vectors comprising such DNA sequences, host cells transformed with such vectors and a process for the production of such muteins employing such transformed host cells and pharmaceutical compositions containing such muteins and their use for the treatment of illnesses, e.g. cancer.

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Tumor Necrosis Factor, or more specifically Tumor Necrosis Factor-alpha, is a cytokine, primarily produced by stimulated macrophages, that exhibits not only a striking cytotoxicity against various tumour cells [Carswell et al., *Proc. Nat. Acad. Sci., U.S.A.* **72**, 3666-3670, (1975)] but also plays a multiple role as a mediator of inflammation and the immune response [for an overview see Beutler and Cerami, *Ann. Rev. Immunol.* **7**, 625-655 (1989); Bonavita and Granger (eds.) "Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy, Karger, Basel (1990)]. The primary structure of human Tumor Necrosis Factor-alpha (hTNF- $\alpha$ ) has been deduced from the nucleotide sequence of a cDNA which has been cloned and expressed in *E. coli* [Pennica et al., *Nature* **312**, 724-729 (1984); Marmenout et al., *Europ. J. Biochem.* **152**, 515-522 (1985); Wang et al., *Science* **228**, 149-154 (1985); Shirai et al., *Nature* **313**, 803-806 (1985)]. A striking homology in amino acid sequence (30%) was found between hTNF- $\alpha$  and human Lymphotoxin, often referred to as human Tumor Necrosis Factor-beta (hTNF- $\beta$ ), a cytokine produced by a subset of lymphocytes [Gray et al., *Nature* **312**, 721-724 (1984); Fiers et al., *Cold Spring Harbour Symp.* **51**, 587-595 (1986)].

hTNF- $\alpha$  with modified amino acid sequences, so called TNF- $\alpha$ -muteins, have also been described in the art [for example see Yamagishi et al., *Protein Engineering* **3**, 713-719, (1990) or by Fiers in "Tumor Necrosis Factors: Structure, Function and Mechanism of Action", Aggarwal and Vilcek (eds.), Marcel Dekker, Inc., New York, in press, or by Fiers et al. in Bonavita and Granger, pp. 77-81 (s.a.)]. In addition TNF- $\alpha$ -muteins have also been the object of several patent applications, e.g. International Patent Applications Publ. Nos. WO 86/02381, WO 86/04606, WO 88/06625 and European Patent Applications Publ. Nos. 155,549; 158,286; 168,214; 251,037 and 340,333, and Deutsche Offenlegungsschrift Nr. 3843534.

Muteins of Lymphotoxin have also been disclosed in the art, e.g. in European Patent Applications Publ. Nos. 250,000; 314,094 and 336,383.

The biological effects of TNF are mediated via specific receptors, namely a receptor with an apparent molecular weight of 55 kD on sodium dodecylsulfate polyacrylamid gel electrophoresis (SDS-PAGE) (p55-TNF-R) and a receptor with an apparent molecular weight of 75 kD on SDS-PAGE (p75-TNF-R). Both forms of TNF-receptors have been cloned, namely p55-TNF-R by Loetscher et al. [*Cell* **61**, 351-359, (1990)] and p75-TNF-R by Dembic et al. [*Cytokine* **2**, 53-58, (1990)] (for both receptors see also European Patent Application No. 90116707.2) and it was found more recently that both receptors bind not only TNF- $\alpha$  but also TNF- $\beta$  with high affinity [Schönfeld et al., *J. Biol. Chem.* **266**, 3863-3869 (1991)].

Object of the present invention is a mutein or a pharmaceutically acceptable salt thereof on the basis of the amino acid sequence of human Tumor Necrosis Factor which sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and the human p55-Tumor-Necrosis-Factor-Receptor.

A preferred embodiment of the present invention is a mutein as defined above on the basis of the amino acid sequence of TNF- $\alpha$  as disclosed by Pennica et al. [s.a.], namely:

```

      1                               10
40  VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

      20                               30
    VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

      40
45  ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP

```

50

55

50 60  
 ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER  
 70  
 5 GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU  
 80 90  
 LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS  
 10 100  
 VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR  
 110 120  
 15 PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU  
 130  
 GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU  
 140 150  
 20 ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL  
 157  
 TYR PHE GLY ILE ILE ALA LEU

25 or as disclosed by Marmenout et al. (s.a.) or Wang et al. (s.a.) or Shirai et al. or more specifically as coded for by the nucleotide sequence of the insert of the plasmid pDS56/RBSII, Sph1-TNF $\alpha$  (see Figures 3a and 3b and Example I) coding for mature TNF- $\alpha$ .

30 A specifically preferred embodiment of the present invention is a mutein as defined above wherein the TNF- $\alpha$  amino acid sequence is changed by substitution of one or more amino acids, preferably one or two by other amino acids, preferably naturally occurring amino acids.

35 More specifically preferred embodiments of the present invention are muteins as defined above wherein the TNF- $\alpha$  amino acid sequence is substituted at position 29 and/or 32 or position 31 and 32 or position 31 or position 29 and 31 whereby substitutions at position 29 and/or 32 or position 31 and 32 or position 31 are preferred (referring to a TNF- $\alpha$  amino acid sequence with 157 amino acids) by other amino acids, preferably naturally occurring amino acids. Any amino acid, preferably any naturally occurring one, can be used at this position which leads to a TNF-mutein showing a significant difference between its binding affinity to the human p75-TNF-R and the human p55-TNF-R, whereby for substitutions at position 29 serine, glycine or tyrosine are preferred whereby serine is especially preferred, e.g. in case of a single position
 40 mutein at position 29 (Ser<sup>29</sup>-TNF $\alpha$ ). For substitutions at position 31 glutamic acid, e.g. Glu<sup>31</sup>-TNF $\alpha$ , or asparagine are preferred. For substitutions at position 32 tyrosine, e.g. Tyr<sup>32</sup>-TNF $\alpha$  or tryptophan, e.g. Trp<sup>32</sup>-TNF $\alpha$  are preferred, whereby the latter one is specifically preferred. Especially preferred substitutions in case of a double position mutein at positions 29 and 32 are Ser<sup>29</sup>-Trp<sup>32</sup>-TNF $\alpha$  and at position 31 and 32 are Asn<sup>31</sup>-Thr<sup>32</sup>-TNF $\alpha$ . It is understood that the muteins of the present invention can also be prepared by
 45 methods known in the art of chemical peptide and protein synthesis, e.g. by partial or total liquid or solid phase synthesis as described e.g. by Gross and Meyenhofer in "The Peptides" Vols. 1-9, Academic Press, Inc., Harcourt Brace Jovanovich, Publs., San Diego (1979-1987) or by Fields and Nobel, Int. J. Pept. Prot. Res 35, 161-214 (1990).

50 Analogs obtained by deletion, substitution and/or addition of one or several amino acids from or to the muteins as defined in the previous paragraph whereby position 29 and/or 32 or position 31 or position 31 and 32 in the mutein is/are not changed and which analogs still show a significant difference between its binding affinity to the human p75-TNF-R and the human p55-TNF-R are also an object of the present invention. With respect to such substitution analogs amino acid substitutions in proteins which do not generally alter the activity are known in the state of the art and are described, for example, by H. Neurath
 55 and R.L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially Figure 6, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr. Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse (the three letter abbreviations are used for amino acids and are standard and known in the art).

Substitution, addition and/or deletion analogs can be produced by methods known in the art and described e.g. in Sambrook et al. [Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbour Laboratory, Cold Spring Harbour Laboratory Press, USA (1989)] or in the following paragraphs. Whether such an analog still shows the significant difference between its binding affinity to the p75-TNF-R and the p55-TNF-R can be determined as described in the following and e.g. more specifically in Examples IIa) and b) or Example VIII. Furthermore salts of such muteins and analogs are also an object of the present invention. Such salts can be produced by methods known in the art.

It is furthermore an object of the present invention to provide a mutein as described above for the treatment of illnesses, e.g. cancer.

It is well known in the art that on the basis of its biological activities (s.a.) TNF- $\alpha$  can be a valuable compound for the treatment of various disorders. For example TNF- $\alpha$ , alone or in combination with interferon, can be an effective antitumor agent [Brouckaert et al., Int. J. Cancer 38, 763-769 (1986)]. However, its systemic toxicity is a major limitation to its wider therapeutic use [Taguchi T. and Sohmura Y., Biotherapy 3, 177-186 (1991)].

The discovery of two TNF-receptors with (putatively) distinct functional roles should allow to dissect in a given disease state the beneficial and unwanted biological responses to TNF. There is circumstantial evidence supporting the feasibility of this approach. It has been shown for example [Brouckaert et al., Agents and Actions 26, 196-197 (1989); Everaerd, B. et al., Biochem. Biophys. Res. Comm. 163, 378-385 (1989)] that in mice murine TNF- $\alpha$  (mTNF- $\alpha$ ) is up to 50-fold more toxic than human TNF- $\alpha$  (hTNF- $\alpha$ ), although when tested in cell culture, both are equally active on sensitive cell lines.

It is believed that the strategy of dissecting beneficial and unwanted TNF $\alpha$  activities by using compounds specifically binding to one or the other TNF-receptor, such as the TNF-muteins of the present invention, can be used in general in other disease states where TNF plays a role.

DNA-sequences comprising a DNA-sequence coding for TNF-muteins as hereinbefore described are also an object of the present invention. Such DNA-sequences can be constructed starting from genomic- or cDNA-sequences coding for hTNF as disclosed in the art [s.a.] using known methods of in vitro mutagenesis [see e.g. Sambrook et al., 1989]. Such mutagenesis can be carried out ad-random in order to obtain a large number of mutants which can then be tested for their desired properties in appropriate assay systems or, in order to mutate defined positions in a given DNA-sequence, by so called site directed mutagenesis [see e.g. Sambrook et al., 1989, 15.51-15.113] or by mutagenesis using the polymerase chain reaction [see e.g. White et al., Trends in Genetics 5, 185-189 (1989)].

One chemical mutagen which is often used for mutagenesis ad-random is sodium bisulfite which converts a cytosin residue into an uracil residue and hence leads to a transition of "C" to "T" (standard abbreviations for nucleotides) [for the method see e.g. Shortle and Nathans, Proc. Nat. Acad. Sci. U.S.A. 75, 2170-2174 (1978) or Pine and Huang, Meth. Enzym. 154, 415-430 (1987)]. This mutagen acts solely on single stranded DNA whereas the expression of the mutated target DNA sequence is achieved with a double stranded plasmid vector. One possibility to avoid the necessity of recloning in mutagenesis and expression vectors is the use of so called "phasmsids". These are vectors which, in addition to a plasmid origin of replication, carry also an origin of replication derived from a filamentous phage. Examples of such phasmsids are the pMa- and pMc-phasmsids as described by Stanssen et al. [Nucleic Acids Res. 17, 4441-4454, (1989)]. Using this expression system one can construct so called "gap-duplex"-structures [see also Kramer et al., Nucl. Acids. Res. 12, 9441-9456 (1984)] where only the TNF-coding sequence (s.a.) is in a single stranded configuration and therefore accessible for the specific chemical mutagen. "gap-duplexes" to be used in ad-random mutagenesis can be constructed as described for site-specific mutagenesis by Stanssen et al. [s.a.] with the exception that the (-)strand contains the same active antibiotic resistance gene as the (+)strand. By making use of different restriction sites in the DNA-sequence encoding hTNF $\alpha$ , variation of the width of the gap is possible. Examples of such restriction sites are the C1a1-Sal1 sites (470 nucleotides), BstX1-BstX1 sites (237 nucleotides) or Sty1-Sty1 sites (68 nucleotides). Such gap-duplex-constructs can then be treated with increasing concentrations (up to 4M) of bisulfite, followed by several dialysis steps, as described by Shortle and Nathans (s.a.). A suitable procaryotic host cell can then be transformed by such phasmid constructs according to methods known in the art and described e.g. by Sambrook et al. (s.a.). A suitable procaryotic host cell means in this context a host cell deficient in a specific repair function so that an uracil residue is maintained in the DNA during replication and which host cell is capable of expressing the corresponding mutated TNF. Such specific host strains are known in the art, for example for E. coli strains, e.g. E. coli BW 313 [Kunkel, T.A., Proc. Natl. Acad. Sci. USA 82, 488-492 (1985)]. The resulting clones can then be screened for those expressing a desired TNF-mutein by appropriate assay systems. For example each colony can be inoculated in a microtiterplate in a suitable medium containing the relevant antibiotic. The cells may be lysed by addition of lysozyme, followed by



sequential freeze-thaw cycles. After precipitation of nucleic acids and centrifugation, the supernatant of each colony can directly be used in appropriate assays as described, e.g., in Example IIa and IIb or Example VIII measuring binding to the p75-TNF-R and the p55-TNF-R on the surface of living cells or in purified form.

If desired, the specific sites of mutation can be determined, for example by restriction fragment analysis [see e.g. Sambrook et al. (s.a.)]. By determination of the DNA-sequence of such fragments the exact position of the mutation can be determined and if such mutation leads to an amino acid replacement the new amino acid can be derived from the determined DNA-sequence. DNA-sequencing can be performed according to methods known in the art, e.g. by using T7 polymerase on supercoiled DNA with a commercially available sequencing kit (Pharmacia, Uppsala, Sweden).

As already mentioned above, another possibility of mutating a given DNA-sequence is by "site directed mutagenesis". A widely used strategy for such kind of mutagenesis as originally outlined by Hutchinson and Edgell [J. Virol. 8, 181 (1971)] involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single stranded DNA-sequence wherein the mutation should be introduced [for review see Smith, Annual. Rev. Genet. 19, 423 (1985) and for improved methods see references 2-6 in Stanssen et al. (1989)].

One such preferred method is the one of Stanssen et al. (1989) using "gapped duplex DNA" as originally described by Kramer et al. (1984) [see above and Kramer and Fritz, Methods in Enzymology, (1987), Academic Press, Inc., USA] but using antibiotic resistance genes instead of M13 functional genes for selection of the mutation containing strand in addition with the phasmid-technology as also described by Stanssen et al. (1989) [s.a.]. An advantage of this method lies also in the capability of performing successive cycles of mutagenesis without the need to transfer the gene to a new mutagenesis vector: second round mutagenesis differs only in the selection to another antibiotic marker (Stranssen et al., s.a.). As a control site-specific back mutagenesis of the mutant to the wild-type TNF can be used. In addition, the use of an oligonucleotide, creating or destroying a restriction site in the TNF gene, allows to control the mutant not only by hybridization to the oligonucleotide used for site directed mutagenesis but also by the presence or absence of the restriction site. In order to create a set of TNF-mutants wherein at a defined position of their amino acid sequence the wild-type amino acid is replaced by any naturally occurring amino acid a set of oligonucleotides is used with all possible codons at the defined position.

As already mentioned above, another possibility of mutating a given DNA-sequence is the mutagenesis by using the polymerase chain reaction (PCR). The principles of this method are outlined e.g. by White et al. (1989), whereas improved methods are described e.g. in Innis et al. [PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc. (1990)].

PCR is an *in vitro* method for producing large amounts of a specific DNA fragment of defined length and sequence from small amounts of a template DNA. Thereby, PCR is based on the enzymatic amplification of the DNA fragment which is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are oriented with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. Since the primers are physically incorporated into the amplified product and mismatches between the 5' end of the primer and the template do not significantly affect the efficiency of the amplification, it is possible to alter the amplified sequence thereby introducing the desired mutation into the amplified DNA. By utilizing the thermostable Taq DNA polymerase isolated from the thermophilic bacteria *Thermus aquaticus*, it has been possible to avoid denaturation of the polymerase which necessitated the addition of enzyme after each heat denaturation step. This development has led to the automation of PCR by a variety of simple temperature-cycling devices. In addition, the specificity of the amplification reaction is increased by allowing the use of higher temperatures for primer annealing and extension. The increased specificity improves the overall yield of amplified products by minimizing the competition by non-target fragments for enzyme and primers.

Design and synthesis of oligonucleotides can be effected as known in the art and described e.g. in Sambrook et al. (1989) or in one of the references cited above with respect to site directed mutagenesis.

As soon as a DNA-sequence coding for a TNF-mutagen of the present invention has been created, expression can be effected by the phasmid technology as described above or by use of any suitable pro- or eukaryotic expression system well known in the art [see e.g. Sambrook et al., s.a.].

Expression is effected preferably in prokaryotic cells, e.g., in *E. coli*, *Bacillus subtilis* and so on, whereby *E. coli*, specifically *E. coli* K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 (1974)], HB 101 [ATCC No. 33694], WK6 (Stranssens et al. s.a.) or *E. coli* SG13009

[Gottesman et al., J. Bacteriol. 148, 265-273 (1981)] are preferred. Expression of the muteins of the present invention can also be effected in lower or higher eukaryotic cells, like for example yeast cells (like *Saccharomyces*, *Pichia* etc.), filamentous fungi (like *Aspergillus* etc.) or cell lines (like chinese hamster ovary cell lines etc.), whereby expression in yeast cells is preferred [see Sreekrishna et al., Biochem. 28, 4117-4125, (1989); Hitzeman et al., Nature 293, 717-722 (1981); European Patent Application Publication No. 263 311]. Expression of the TNF-muteins of the present invention may occur in such systems either intracellularly, or, after suitable adaption of the gene, extracellularly (see Leemans et al., Gene 85, 99-108, 1989).

Suitable vectors used for expression in *E. coli* are mentioned e.g. by Sambrook et al. [s.a.] or by Fiers et al. in "Procd. 8th Int. Biotechnology Symposium" [Soc. Franc. de Microbiol., Paris, (Durand et al., eds.), pp. 680-697 (1988)] or and more specifically vectors of the pDS family [Bujard et al., Methods in Enzymology, eds. Wu and Grossmann, Academic Press, Inc. Vol. 155, 416-433 (1987); Stüber et al., Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990)] like for example pDS56/RBSII, Sph1-TNF $\alpha$ Ser29 or pDS56/RBSII, Sph1-TNF $\alpha$ Trp32 (see Example I) or pDS56/RBSII, Sph1-TNF $\alpha$ Glu31 or pDS56/RBSII, Sph1-TNF $\alpha$ Asn31Thr32 (see Example VII). The transformed *E. coli* strains M15 (pREP4;pDS56/RBSII, Sph1-TNF $\alpha$ Glu31) and M15 (pREP4;pDS56/RBSII, Sph1-TNF $\alpha$ Asn31Thr32) have been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, BRD at September 8th, 1991 under accession numbers DSM 6714 and DSM 6715 respectively. Since with these specific pDS56/RBSII-plasmids due to their specific regulatable promoter/operator elements and ribosomal binding sites a high level of expression can be achieved, the plasmids can be maintained in *E. coli* cells only when the activity of the promoter/operator element is repressed by the binding of a lac repressor to the operator. The activity of the promoter can be restored at the desired cell density by addition of IPTG, which inactivates the repressor and clears the promoter. Since most of the *E. coli* strains do not provide enough repressor molecules to completely repress the function of the promoter sequences present in these high copy number plasmids, such *E. coli* strains, like *E. coli* M15 or SG13009, have to be transformed at first with a plasmid, like pREP 4, coding for the lac repressor, before being transformed with the specific pDS56/RBSII-plasmids of the invention which can then be stably maintained in the *E. coli* cells. Beside coding for the lac repressor, pREP4 contains also a region of the plasmid pACYC184 [Chang and Cohen, J. Bacteriol. 134, 1141-1156 (1978)], which contains all information required for replication and stable transmission to daughter cells [for additional information see also "System for high level production in *E. coli* and rapid purification of recombinant proteins: application to epitope mapping, preparation of antibodies and structure function analysis" by Stüber et al. in Immunological Methods, Vol. IV, pp 121-152, Lefkovits and Pernis (eds.), Academic Press, New York (1990)].

Transformation of the host cells by vectors as described above may be carried out by any conventional procedure [see for example Sambrook et al. (s.a.)]. Where the host cell is a prokaryote, such as *E. coli* for example, competent cells which are capable of DNA uptake are prepared from cells harvested after exponential growth phase and subsequently treated according to the known CaCl<sub>2</sub>-method. Transformation can also be performed after forming a protoplast of the host cell or by other methods known in the art and described, e.g., in Sambrook et al. (s.a.). Therefore a vector, especially for expression in a prokaryotic or lower eukaryotic host cell, comprising a DNA-sequence coding for a TNF-mutein as described above, and a host cell, especially a prokaryotic host cell, e.g. *E. coli*, or a lower eukaryotic host cell, transformed by such a vector are also an object of the present invention.

Usually, the host organisms which contain a desired expression vector are grown under conditions which are optimal for their growth. In case of a prokaryotic host at the end of the exponential growth, when the increase in cell number per unit time decreases, the expression of the desired TNF-mutein is induced, i.e. the DNA coding for the desired TNF-mutein is transcribed and the transcribed mRNA is translated. The induction can be carried out by adding an inducer or a derepressor to the growth medium or by altering a physical parameter, e.g. a change in temperature. In the expression vectors used in the preferred embodiments of the present invention, the expression is controlled by the lac repressor. By adding isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), the expression control sequence is derepressed and the synthesis of the desired TNF-mutein is thereby induced.

TNF-muteins of the present invention produced by transformed host cells as stated above can be recovered from the culture medium or after opening the cells and/or extraction by any appropriate method known in protein and peptide chemistry such as, for example, precipitation with ammonium sulfate, dialysis, ultrafiltration, gel filtration or ion-exchange chromatography, gel electrophoresis, isoelectric focusing, affinity chromatography, like immunoaffinity chromatography, HPLC or the like. Specifically preferred methods are precipitation with ammonium sulfate and/or polyethylenimine, dialysis, affinity chromatography, e.g. on

phenyl-agarose, specifically phenyl-sepharose, or ion-exchange chromatography, specifically on a MONO-Q- and/or MONO-S-matrix (Pharmacia, Uppsala, Sweden) or more specifically are those as described by Tavernier et al. [J. Mol. Biol. 211, 493-501 (1990)] and those disclosed in Example I or Example III.

- 5 It is therefore also an object of the present invention to provide a process for the preparation of a compound as specified above which process comprises cultivating a transformed host cell as described above in a suitable medium and isolating a mutein from the culture supernatant or the host cell itself, and if desired converting said mutein into a pharmaceutically acceptable salt. The compounds whenever prepared according to such a process are also an object of the present invention.

- The muteins of the present invention are characterized by showing a significant difference between its  
10 binding affinity to the human p75-TNF-R and the human p55-TNF-R. Such property can be determined by any assay known in the art measuring binding affinities. For example the binding of TNF itself and of the muteins of the present invention can be measured using cells in cell culture which express the two types of TNF-receptors to a different degree, like for example Hep-2 cells which exclusively express the human p55-TNF-R and U937 or HL60 cells which express in addition also the human p75-TNF-R [see Brockhaus et al.,  
15 Proc. Nat. Acad. Sci. U.S.A. 87, 3127-3131, (1990); Hohmann et al., J. Biol. Chem. 264, 14927-14934, (1989); Loetscher et al. (1990); Dembic et al. (1990)]. Of course binding affinities can also be determined directly by using purified native or recombinant p55-TNF-R and p75-TNF-R as specifically described in Example IIb, or by using the corresponding soluble analogs of such receptors.

- The term significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor" refers in the context of the present invention to a difference in binding affinities to the two types of TNF-receptors which is with respect to the used assay system significant enough to say that a mutein of the present invention binds preferentially to one of the two TNF-receptors as compared to wild type TNF. More specifically this term means in the context of the assay-system of Example IIa) that a  $K_D$ -value of a specific TNF-mutein of the present  
25 invention is at least a factor of 10 or more, especially preferred at least a factor of  $10^2$ , larger than for TNF- $\alpha$  itself determined by using U937 cells whereby its  $K_D$ -value determined by using Hep-2 cells for the same TNF-mutein is not larger than a factor of 2 as for TNF- $\alpha$ : itself [for specific data see Table I of Example IIa)]. It is however understood that these specific  $K_D$ -values are given for illustration and should not be considered as limiting in any manner.

- 30 The muteins of the present invention can be characterized by their anti-tumour activity by methods known in the art and described e.g. in Example IV.

The muteins of the present invention may show but not necessarily considerably reduced cytotoxic activity in standard TNF-assays which are based on murine cell lines, such as L929 (see Table 1) or L-M cell lines.

- 35 TNF-muteins of the present invention can be used for the treatment of illnesses. e.g. cancer.

- A further object of the present invention is a pharmaceutical composition and a process for its preparation which composition contains one or more compounds of the invention, if desired in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials. For this purpose, one or more compounds of the invention, where desired or required in  
40 combination with other pharmaceutically active substances, can be processed in a known manner with the usually used solid or liquid carrier materials. The dosage of such preparations can be effected having regard to the usual criteria in analogy to already used preparations of similar activity and structure. After the invention has been described in general hereinbefore, the following Examples are intended to illustrate details of the invention, without thereby limiting it in any manner.

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## Examples

### Example I

- 50 Preparation of Ser<sup>29</sup>-TNF $\alpha$  and Trp<sup>32</sup>-TNF $\alpha$

#### Construction of a mutagenesis vector

- From the human TNF expression plasmid pDS56/RBSII, Sph1-TNF $\alpha$  (see Figure 3a: The expression  
55 plasmid contain the regulatable promoter/operator element N25OPSN25OP29



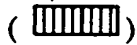
the synthetic ribosomal binding site RBSII



5  
genes



10  
for  $\beta$ -lactamase (bla), chloramphenicol acetyltransferase (cat), and transcriptional terminators



15  
 $t_0$  of phage lambda ( $t_0$ ) and T1 of *rmB* operon of *E. coli* (T1), and the replication region of plasmid pBR322 (repl.). The coding region under control of N25OPSN25OP29 and RBSII is indicated by an arrow; for complete nucleotide sequence of the plasmid see Figure 3b/1-3b/3 given by the one letter standard abbreviations for nucleotides), an EcoRI-HindIII fragment was isolated, containing the ribosome binding site RBSII, the mature TNF $\alpha$  coding sequence and a 130 bp 3' non-translated sequence. This fragment was  
20 cloned into the EcoRI-HindIII opened pMac phasmids (Stanssens et al., s.a.), resulting in the constructions pMa/RBSII,Sph1-TNF $\alpha$  and pMc/RBSII,Sph1-TNF $\alpha$ .

#### Isolation of single-stranded (ss)DNA

25  
The pMa/RBSII,Sph1-TNF $\alpha$  phasmid was transformed to *E. coli* WK6 (Stanssens et al., s.a.). One colony was picked up and cultured in 5 ml LB medium (Sambrook et al., 1989) + carbenicillin (50  $\mu$ g/ml) at 37°C, overnight. 1 ml of this confluent culture was used to inoculate 200 ml LB + carbenicillin. When the absorbance (650 nm) reached a value of 0.1, the culture was infected with M13K07 helper phage  
30 (Stanssens et al., (1989) at a m.o.i. of about 20 and further incubated overnight at 37°C. Then, the cells were spun down (10 min, 10.000 rpm) and the supernatant was transferred into another tube. 50 ml PEG-solution (20% polyethylene glycol 6000; 2.5 M NaCl) was added and the mixture was kept on ice for one hour to precipitate the phages. After centrifugation (10 min; 8000 rpm), the supernatant was removed and the tube was dried on paper towels for 10 min. The phage pellet was resuspended in 6 ml TE buffer (10  
35 mM Tris-HCl, 0.1 mM EDTA, pH8). A first extraction was performed with 6 ml TE-saturated phenol, followed by vortexing for 3 min. After centrifugation (3 min) in an Eppendorf centrifuge, the aqueous phase was transferred to a fresh tube and a second extraction was carried out with chloroform:isoamylalcohol (24:1), the same way as described. The single stranded DNA could be precipitated by adding 1/10 volume of 5M NaClO<sub>4</sub> and 1 volume of isopropanol (-20°C, 2 hours). This ssDNA was pelleted by centrifugation for  
40 20 min in an Eppendorf centrifuge. The pellet was dried and dissolved in 500  $\mu$ l TE buffer as a control, 5  $\mu$ l of this mixture was run on an agarose gel, containing 1  $\mu$ g/ml ethidium bromide. Usually, the ratio of pMa/RBSII,Sph1-TNF $\alpha$  ssDNA (= (+)strand) over helper phage ssDNA was between 2:1 and 20:1. The amount of total ssDNA was estimated to be at least 200 ng/ $\mu$ l.

#### 45 Construction of a gap-duplex

From the phasmid pMc, the EcoRI-HindIII large fragment was isolated and used for hybridization to the pMa/RBSII, Sph1-TNF $\alpha$ (+)strand. In a typical experiment, 15  $\mu$ l ssDNA ( $\pm$  3  $\mu$ g), 15  $\mu$ l of the double  
50 stranded, linear fragment ( $\pm$  1.5  $\mu$ g), 10 ml hybridization buffer (1.5 M KCl; 100 mM Tris-HCl, pH 7.5) and 40  $\mu$ l H<sub>2</sub>O were mixed and incubated at 100°C for 4 min, 65°C for 8 min and room temperature for 15 min. An aliquot (10 ml) was electroforesed on an agarose gel containing ethidium bromide, to check the formation of gap duplex DNA and, if so, to estimate its quantity (this usually amounted to 50ng/10ml hybridization mixture). Annealing of the mutant oligonucleotide and fill-in of the gap duplex Oligonucleotides were synthesized containing the mutated codon and destroying or creating a restriction site in the TNF  
55 gene. The oligonucleotides 5'CCGGCGGTTGGACCACTGGAGC3' and 5'CATTGGCCCAGCGGTTTCAG3' (mutated bases underlined) were used to create the Ser<sup>29</sup> and Trp<sup>32</sup> mutations respectively. After enzymatic phosphorylation, about 8 pmol was added to 40 ng of gap-duplex. H<sub>2</sub>O was added to a final volume of 10 ml. This mixture was heated to 65°C for 5 min and allowed to cool to room temperature. Subsequently, 18

ml H<sub>2</sub>O, 4 µl fill-in buffer 10 (625 mM KCl, 275 mM Tris-HCl, 150 mM MgCl<sub>2</sub>, 20 mM DTT pH 7.5), 2 µl ATP 1mM, 4 µl of the four dNTP's 1mM, 1 µl ligase and 1 ml Klenow polymerase were added and the mixture was incubated at room temperature for 45 min.

#### 5 Transformation to E. coli WK6 mutS and E. coli WK6

We used 10 µl of the filled-in gap duplex DNA to transform (Sambrook et al., 1989) E. coli WK6 mutS (Strassens et al., s.a.). From this mixture (1.2 ml), 100 µl was plated out on agar plates containing 25 µg/ml chloramphenicol to check transformation efficiency. The remainder was used to inoculate 20 ml LB + chloramphenicol and further grown overnight at 25 °C. A small-scale plasmid DNA preparation [Birnboim, H.C. and Doly, J., Nucleic Acids Res., 7, 1513, (1979)] of this culture (not yet grown to confluency) resulted in a mixed phasmid population that could be transformed to E. coli WK6. Again, 100 µl transformation mixture was plated out on agar plates containing chloramphenicol.

#### 15 Screening by colony hybridization

About 100 colonies, resulting from the transformation to E. coli WK6, were streaked on a nylon filter (PALL, Glen Cove, New York) and incubated overnight at 37 °C. The filter was transferred (face up) to Whatmann 3MM papers which were soaked in 0.5 M NaOH (3 min). Neutralization was done by transfer to Whatmann 3MM sheets soaked in 1M Tris-HCl pH 7.4 (twice for 1 min) and 2XSSC (20XSSC=3M NaCl; 0.3M Na citrate, pH7) (5 min). After drying, the filter was baked at 80 °C between sheets of 3MM paper. Subsequently, the filter was prewetted in 6xSSC (5 min) and prehybridized at 67 °C for 5 min in 10x Denhardt solution (2% (w/v) Fico11 (400,000 MV), 2% (w/v) Polyvinylpyrrolidone (44,000 MW), 2% (w/v) Bovine Serum Albumin), 6xSSC buffer and 0.2% SDS. After rinsing in 6xSSC buffer, the filter was placed in a Petri dish containing 4 ml 6xSSC and 60 pmol of the <sup>32</sup>p-labeled mutant oligonucleotide for 1 hour at room temperature, and rinsed in 100 ml 6xSSC. The filter was covered with Saranwrap and autoradiographed on preflashed films (Fuji) at -70 °C for 1 hour. Subsequently, the filter was again washed in 6xSSC buffer at increasing temperatures (varying between 51 °C and 75 °C, according to the length of the probe and its amount of G and C residues), followed each time by an autoradiography, as described above. For instance, a wash at 64 °C could clearly distinguish the Ser29 mutants from the wild-type colonies, while the Trp32 mutants were detected after two subsequent washes at 62 °C and 63 °C, respectively.

#### Restriction fragment analysis

Because the Ser29 mutation created an Ava2 restriction site and Arg32 destroyed the Nci1 restriction site, both corresponding endonucleases could be used for restriction fragment analysis to check once again the presence of the mutation. The colonies were picked up and grown to confluency in 5 ml + chloramphenicol. From these cultures, plasmid DNA was prepared, digested with the appropriate restriction endonucleases and electrophoresed on agarose gels, according to classical procedures (Sambrook et al., 1989).

#### Subcloning to a bacterial expression vector

Transfer of the mutated TNF gene to an expression vector was carried out exactly the opposite way as the construction of the mutagenesis vector. The phasmid pMc/RBSII,Sph1-TNFαSer29 or pMc/RBSII,Sph1-TNFαTrp32 was digested with EcoR1-HindIII and the small fragment was inserted into the EcoR1-HindIII opened pDS56/RBSII,Sph1-TNFα vector generating plasmids pDS56/RBSII,Sph1-TNFαSer29 and pDS56/RBSII,Sph1-TNFαTrp32 and transformed into E. coli M15 cells containing already plasmid pREP4 (encoding the lac repressor; see Figures 2a and 2b/1-2b/3 for a complete nucleotide sequence of the plasmid given by the one letter standard abbreviations for nucleotides) by standard methods (s.a.). Such cultures of transformed E. coli M15 were grown at 37 °C in LB medium (10 g bacto tryptone, 5 g yeast extract, 5 g NaCl per litre) containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2mM. After additional 2.5 to 5 h at 37 °C the cells were harvested by centrifugation and the TNF muteins were purified according to Tavernier et al. [J. Mol. Biol. 211, 493-501, (1990)]. The transformed E. coli strains M15 (pREP4;pDS56/RBSII,Sph1-TNFαSer29) and M15(pREP4;pDS56/RBSII,Sph1-TNFαTrp32) have been deposited under the Budapest Treaty for patent purposes at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH(DSM) in Braunschweig, BRD at November 19th, 1990 under accession numbers DSM 6240 and DSM 6241

respectively.

## Example II

### 5 Characterization of Ser<sup>29</sup>-TNF $\alpha$ and Trp<sup>32</sup>-TNF $\alpha$

#### a) Differential binding and biological activity on Hep2- and U937 cells

##### Cell culture

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Hep-2 [ATCC No. CCL 23], U937 [ATCC No. CRL 1593] and RAJI [ATCC No. CCL 86] cells were grown in RPMI 1640 medium, supplemented with 10% (v/v) inactivated fetal calf serum, L-glutamine (2mM), sodium pyruvate (1mM), 2-mercaptoethanol ( $5 \times 10^{-5}$  M), 1% of a 100x mixture of non-essential amino acids [Gibco Laboratories, Paisley, GB] and gentamycine (25 mg/ml). The non-adherent cells (U937 and RAJI) were harvested after reaching a density of  $1 \times 10^6$  cells/ml. For binding experiments, the adherent Hep-2 cells were grown to confluency, trypsinized, collected and seeded in large Petri dishes (150 cm<sup>2</sup>) at a density of  $2.5 \times 10^6$  cells/ml. Subsequently, the dishes were placed in a CO<sub>2</sub>-incubator overnight. Because Hep-2 cells are not strongly adherent, the cells could be harvested the same way as the non-adherent cells. Dulbecco's medium, supplemented with 10% inactivated newborn calf serum was used for L929 cell growth.

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##### Determination of the specific activities on L929, Hep-2 and U937 cells.

The amount of protein was determined by the Biorad (Richmond, CA, USA) protein dye reagent according to the instructions of the manufacturer. The purity of the TNF muteins was determined by SDS-PAGE.

The cytotoxic activity on mouse L929 cells was determined using the standard L929 assay (Ruff and Gifford in "Lymphokines", ed. by E. Pick, Vol. 2, 235-275, Academic Press, 1981, Orlando, USA). The cytotoxicity assay on Hep-2 cells was performed the same way as the L929 assay with the only exception that cycloheximide (50  $\mu$ g/ml) was added instead of actinomycin D.

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##### Receptor binding assay

##### -Iodination of TNF- $\alpha$ and Trp<sup>32</sup>-TNF

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5  $\mu$ g Iodogen (Pierce, USA) was dissolved in 10  $\mu$ l chloroform and dried under a nitrogen stream in a small glass tube. To this, 10  $\mu$ l Na<sup>125</sup>I (Amersham, 100 mCi/ml in 0.1 M borate buffer, pH 8) was added and kept for 15 min. on ice. This solution was quickly pipetted to an Eppendorf tube, containing 5  $\mu$ g TNF- $\alpha$  - [Pennica et al., s.a.] or 3.2  $\mu$ g of Trp<sup>32</sup>-TNF in 10  $\mu$ l phosphate buffer pH 7. Again the reaction was kept for 15 min on ice. To separate the iodinated TNF- $\alpha$  from the Na<sup>125</sup>I, a PD-10 gelfiltration column (Pharmacia) was first equilibrated with 0.1 M phosphate buffer + 0.25% gelatin and prerun with 1  $\mu$ g TNF- $\alpha$  or Trp<sup>32</sup>-TNF, depending on the iodinated TNF species. Subsequently, the reaction mixture was loaded onto the column, and fractions of about 400  $\mu$ l were collected from which 2  $\mu$ l aliquots were counted in a  $\gamma$ -counter (LKB 1275 Minigamma, Pharmacia LKB, Uppsala, Sweden). A specific radioactivity of 10-75 and 80  $\mu$ Ci/mg was obtained for THF- $\alpha$  and Trp<sup>32</sup>-TNF, respectively.

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##### -Determination of the K<sub>D</sub>-value of labeled TNF- $\alpha$ and Trp<sup>32</sup>-TNF by Scatchard analysis

A dilution series in steps of factor 2 in the range of 12.8nM  $\rightarrow$  0.006nM of the labeled TNF- $\alpha$  or Trp<sup>32</sup>-TNF was made up in a microtiterplate. Each dilution was made in triplicate. Non-specific binding was measured by the same setup, wherein each point contained a 100 fold excess of unlabeled TNF (1.28  $\mu$ M  $\rightarrow$  0.6nM). To each well, approximately  $2 \times 10^6$  cells (U937, Hep-2 or RAJI) were added. The reaction was performed in 0.2 ml tissue culture medium, containing 0.1% NaN<sub>3</sub> for 2-3 hours at 4 °C. After this, samples were transferred from the microtiterplates to small plastic tubes (Micronic systems), already containing 300  $\mu$ l phthalate oil (dinonylphthalate 33%, dibutylphthalate 66% (v/v)). The tubes were centrifuged in a microfuge (Eppendorf) for 10 min. to spin down the cells, thereby separating them from the supernatant, using the phthalate oil as a separation medium. After inversion of the tubes, the cell pellet (now on top) could easily be isolated by melting off the top of the tubes with a hot scalpel. The amount of radioactivity,

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bound on the cells, was measured by counting in a  $\gamma$ -counter. From these data, a Scatchard plot and, subsequently, the dissociation constant  $K_D$  was determined using the equilibrium binding type "HOT" in the EBDA/LIGAND program [McPherson et al., J. Pharmacol. Methods 14, 213-228, (1985)].

5 -Determination of the  $K_D$  of mutant TNF [Ser<sup>29</sup>-TNF- $\alpha$  and Trp<sup>32</sup>-TNF- $\alpha$ ] by competition analysis

The Scatchard data showed that a concentration of 0.4 nM radiolabeled TNF- $\alpha$  was high enough to show a clearly detectable signal and fell within the linear part of the saturation curves. This concentration, however, was also low enough to allow addition up to a 5000 fold excess of cold mutant TNF (2  $\mu$ M),  
 10 necessary to perform a competition experiment in which <sup>125</sup>I-wild type TNF is the primary ligand and cold mutant the competitor.

A ten well dilution series of unlabeled mutant TNF (2 mM  $\rightarrow$  0.004  $\mu$ M) in concentration steps of factors x2 was set up in a microtiterplate. The two remaining wells contained no unlabeled TNF (total binding) and a 5000 fold excess of the wild-type, unlabeled TNF (background), respectively. To all wells, 0.4 nM of radiolabeled TNF- $\alpha$  (10-75  $\mu$ Ci/ $\mu$ g) was added. After addition of  $2 \times 10^6$  cells, the total volume was 0.2 ml/well. Medium of incubation, reaction conditions and isolation of the cells were exactly the same as described above for the Scatchard analysis experiments. Each point was measured in triplicate and the dissociation experiments were done twice, the average of the two  $K_D$ 's being indicated in Table 1. Using the "DRUG" method of the EBDA/LIGAND program (s.a.), competition curves were plotted and the  $K_D$  of the  
 20 mutants was calculated. The following experimental data were used for such calculations:

1. Labeling of hTNF

first labeling (= batch 1): 1.2x10<sup>8</sup> dpm/5 $\mu$ g  
 25 = 3.7x10<sup>5</sup> dpm/pmol  
 =  $\pm 10 \mu$ Ci/ $\mu$ g  
 second labeling (= batch 2:) 5.3x10<sup>8</sup> dpm/3.2  $\mu$ g  
 = 1.9x10<sup>6</sup> dpm/pmol  
 =  $\pm 75 \mu$ Ci/ $\mu$ g  
 30

2. Determination of the  $K_D$  of wild-type TNF

We measured the  $K_D$  of <sup>125</sup>I-TNF (batch 1) on Hep-2 and U937 cells by Scatchard analysis.

Hep-2:  $K_D = 9.17 \times 10^{-10}$   
 35 U937:  $K_D = 2.5 \times 10^{-10}$

3. Competition experiments

All displacement experiments were carried out, using <sup>125</sup>I-TNF (batch 1) as the primary ligand, except  
 40 experiment B.3 (table B, 3.), where <sup>125</sup>I-TNF (batch 2) was used.

In each experiment, the binding at each concentration was measured in triplicate and only the averages are shown in the following tables (A-D).

From each experiment shown in these tables, the  $K_D$  value was calculated using the program of Mc. Pherson et al. (1985). The average of the  $K_D$  determinations (2 experiments for Ser<sup>29</sup>-TNF $\alpha$  on Hep-2 cells  
 45 and on U937 cells, two experiments for Trp<sup>32</sup>-TNF $\alpha$  on Hep-2 cells and three on U937 cells) are shown in table 1.

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Table ACompetition with. Ser<sup>29</sup>-TNF $\alpha$  on U937 cells.

5	Mean dpm	concentration of mutant [mol]
	1. 2120	0
10	1869	$1 \times 10^{-9}$
	1779	$2 \times 10^{-9}$
	1719	$4 \times 10^{-9}$
	1708	$8 \times 10^{-9}$
15	1575	$1.6 \times 10^{-8}$
	1415	$3.2 \times 10^{-8}$
	1320	$6.4 \times 10^{-8}$
20	1200	$1.25 \times 10^{-7}$
	983	$2.5 \times 10^{-7}$
	949	$5 \times 10^{-7}$
25	632	$1 \times 10^{-6}$
	533	$2 \times 10^{-6}$
	Background: 299	

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	2.	1014	0
		635	$4 \times 10^{-9}$
5		603	$8 \times 10^{-9}$
		541	$1.5 \times 10^{-8}$
		572	$3 \times 10^{-8}$
10		489	$6 \times 10^{-8}$
		413	$1.2 \times 10^{-7}$
		380	$2.5 \times 10^{-7}$
		319	$5 \times 10^{-7}$
15		263	$1 \times 10^{-6}$
		238	$2.1 \times 10^{-6}$
	Background:	205	

Table BCompetition with Trp<sup>32</sup>-TNF- $\alpha$  on U937 cells

	1.	2120	0
		1917	$1 \times 10^{-9}$
25		1698	$2 \times 10^{-9}$
		1655	$4 \times 10^{-9}$
		1585	$8 \times 10^{-9}$
30		1488	$1.5 \times 10^{-8}$
		1377	$3 \times 10^{-8}$
		1333	$6 \times 10^{-8}$
35		1166	$1.25 \times 10^{-7}$
		1026	$2.5 \times 10^{-7}$
		953	$5 \times 10^{-7}$
		777	$1 \times 10^{-6}$
40		628	$2 \times 10^{-6}$
	Background:	299	
	2.	1047	0
45		653	$4 \times 10^{-9}$
		629	$8 \times 10^{-9}$
		636	$1.5 \times 10^{-8}$
50		585	$3 \times 10^{-8}$
		546	$6 \times 10^{-8}$

55

		508	$1.2 \times 10^{-7}$
		479	$2.5 \times 10^{-7}$
5		422	$5 \times 10^{-7}$
		357	$1.1 \times 10^{-6}$
		294	$2 \times 10^{-6}$
10	Background:	214	
	3.	8340	0
	(carried out	4759	$4 \times 10^{-9}$
15	with $^{125}\text{I}$ -	4041	$8 \times 10^{-9}$
	TNF, batch 2)	3620	$1.5 \times 10^{-8}$
		3275	$3 \times 10^{-8}$
		3034	$6 \times 10^{-8}$
20		2387	$1.25 \times 10^{-7}$
		1981	$2.5 \times 10^{-7}$
		1472	$5 \times 10^{-7}$
25		1192	$1 \times 10^{-6}$
		814	$2 \times 10^{-6}$
	Background:	307	

Table CCompetition with Ser<sup>29</sup>-TNF- $\alpha$  on Hep-2 cells

	1.	938	0
		799	$1 \times 10^{-9}$
35		677	$2 \times 10^{-9}$
		564	$4 \times 10^{-9}$
		510	$8 \times 10^{-9}$
40		451	$1.6 \times 10^{-8}$
		442	$3.2 \times 10^{-8}$
		446	$6.4 \times 10^{-8}$
		379	$1.25 \times 10^{-7}$
45		374	$2.5 \times 10^{-7}$
		437	$5 \times 10^{-7}$
		359	$1 \times 10^{-6}$
50		383	$2 \times 10^{-6}$
	Background:	353	

	2.	457	0
		273	$4 \times 10^{-9}$
5		240	$8 \times 10^{-9}$
		253	$1.5 \times 10^{-8}$
		235	$3 \times 10^{-8}$
10		207	$6 \times 10^{-8}$
		239	$1.2 \times 10^{-7}$
		215	$2.5 \times 10^{-7}$
		211	$5 \times 10^{-7}$
15		193	$1 \times 10^{-6}$
		238	$2 \times 10^{-6}$
	Background:	215	

Table D

Competition with Trp<sup>32</sup>-TNF- $\alpha$  on Hep-2 cells

25	1.	938	0
		742	$1 \times 10^{-9}$
		608	$2 \times 10^{-9}$
30		537	$4 \times 10^{-9}$
		547	$8 \times 10^{-9}$
		397	$1.6 \times 10^{-8}$
		394	$3.2 \times 10^{-8}$
35		405	$6.4 \times 10^{-8}$
		395	$1.25 \times 10^{-7}$
		388	$2.5 \times 10^{-7}$
40		379	$5 \times 10^{-7}$
		353	$1 \times 10^{-6}$
		386	$2 \times 10^{-6}$
	Background:	353	
45			
	2.	445	0
		298	$4 \times 10^{-9}$
50		222	$8 \times 10^{-9}$
		256	$1.5 \times 10^{-8}$

	202	$3 \times 10^{-8}$
	227	$6 \times 10^{-8}$
5	210	$1.2 \times 10^{-7}$
	221	$2.5 \times 10^{-7}$
	197	$5 \times 10^{-7}$
10	231	$1 \times 10^{-6}$
	202	$2 \times 10^{-6}$
	Background: 203	

Table 1

	<u>Hep-2</u>		<u>U937</u>	<u>L929</u>
	affinity	specific activity	affinity	specific activity
	(K <sub>D</sub> )	(U/mg)	(K <sub>D</sub> )	(U/mg)
25	TNF- $\alpha$ $9.17 \times 10^{-10} (*)$	$2.9 \times 10^7$	$2.5 \times 10^{-10} (*)$	$2 \times 10^7$
	(100%)	(100%)	(100%)	(100%)
	Ser <sup>29</sup> - $1.06 \times 10^{-9}$	$9.3 \times 10^6$	$5.07 \times 10^{-8}$	$10^5$
30	TNF- $\alpha$ (86.5%)	(32%)	(0.49%)	(0.5%)
	Trp <sup>29</sup> - $1.06 \times 10^{-9}$	$4.5 \times 10^7$	$3.53 \times 10^{-8}$	$6.4 \times 10^4$
	TNF- $\alpha$ (86.5%)	(155%)	(0.71%)	(0.32%)

K<sub>D</sub> values indicated by an asterisk (\*) were obtained by Scatchard analysis. All other K<sub>D</sub> values were determined by competition analysis. Relative values (in percentage to TNF- $\alpha$ ) are indicated between brackets.

It can be seen that the binding constant (K<sub>D</sub>) of Ser<sup>29</sup>-TNF- $\alpha$  and Trp<sup>32</sup>-TNF- $\alpha$  determined with Hep-2 cells (which only carry the p55-TNF-R) are almost the same as the one of TNF- $\alpha$ . Also the biological activity (specific activity) on these cells is largely retained (note that the accuracy of this assay is only a factor 3). Strikingly, the binding affinity (measured in the competition assay) of Ser<sup>29</sup>-TNF- $\alpha$  and Trp<sup>32</sup>-TNF- $\alpha$  to the U937 cells, which predominantly - but not exclusively - carry the high affinity receptor p75-TNF-R, has been largely lost (increase in K<sub>D</sub>-value by a factor of more than 100). It may also be noted that the biological activity of Ser<sup>29</sup>-TNF- $\alpha$  and Trp<sup>32</sup>-TNF- $\alpha$ , determined in the standard assay based on L929-cells, has been largely lost (decrease by a factor more than 100).

b) Differential binding to the human p75-TNF-R and the human p55-TNF-R.

Competition of human <sup>125</sup>I-TNF- $\alpha$  binding by Trp<sup>32</sup>- and Ser<sup>29</sup>-TNF- $\alpha$  and human TNF- $\alpha$  to TNF-receptors purified from HL60 cells was determined as follows. 2  $\mu$ l aliquots of the native p55-TNF-R and the p75-TNF-R purified as described in European Patent Application No. 90116707.2 dissolved at a concentration of about 0.3 mg/ml in 20 mM Hepes, 50 mM Tris, 50 mM NaCl, 1 mM EDTA, 0.1% octylglucoside, 0.1 mg/ml BSA, pH 8.0, were spotted onto prewetted nitrocellulose filters in triplicate. The filters were blocked with blocking buffer (50 mM Tris, 140 mM NaCl, 5 mM EDTA, 0.02% NaN<sub>3</sub>, 1% defatted milk powder) for 1.5 hours at room temperature. After washing with PBS the filters were incubated with 10 ng/ml <sup>125</sup>I TNF $\alpha$  and varying concentrations of Trp<sup>32</sup>- or Ser<sup>29</sup>-TNF $\alpha$ , or TNF $\alpha$  overnight at 4 °C. The filters were washed with blocking buffer (2x for 5 min.) and with H<sub>2</sub>O (1x for 5 min.), air dried, and counted in a  $\gamma$ -counter. Results

are given in Figures 1a and b, whereby Figure 1 shows binding of  $\text{TNF}\alpha$  (open rectangle),  $\text{Ser}^{29}\text{-TNF}\alpha$  - (filled circles) and  $\text{Trp}^{32}\text{-TNF}\alpha$  (filled rectangle) to human p75TNF-R in case of Figure 1a to human p75-TNF-R and in case of Figure 1b to human p55-TNF-R.

### 5 Example III

#### Purification of $\text{Trp}^{32}\text{-TNF}\alpha$

Transformed cells obtained according to Example I were processed in the following manner:

- 10 a) Opening by French press, addition of polyethylene-imine until a final concentration of 0.4%, pH 7.6; removal of precipitate.
  - b) Ammonium sulphate precipitation at pH 7.2; fraction 30-70%
  - c) Dialysis against 25% ammonium sulphate in 10 mM Tris, pH 6.8
  - d) Phenyl-Sepharose column CL-4B (35 x 250 mm)
  - 15 Load in 25% ammonium sulphate - 10 mM Tris, pH 6.8  
Elution: gradient 25% ammonium sulphate-Tris buffer to 20 mM ethanolamine, pH 9 (2 times 150 ml).
  - e) Column Mono Q (HR 16/10).  
Load: in 20 mM ethanolamine, pH 9. Elution: gradient (2 times 300 ml) in the same buffer, from 0 to 1 M sodium chloride (Pharmacia, FPLC). Active fractions dialysed versus 0.01 M phosphate buffer pH 7
  - 20 f) Column of Heparin Sepharose CL-6B (30 x 80 mm)  
Load in 0.01 M phosphate buffer pH 7. Elute with a gradient in the same buffer from 0 to 1 M sodium chloride
  - g) Active fractions were concentrated on Amicon (micro-ultrafiltration system 8 MC; membrane O 25 mm; diaflo 10 YM10 - 25 mm) and separately loaded on a gelfiltration column (Ultrapac TSK G-2000 SWG; 25 21.5 x 600 mm), equilibrated in 0.01 M phosphate pH 7 and 0.9% sodium chloride
- LPS (determined by test kit of Kabivitrum):  
Most active fraction contained 5 mg/ml  $\text{Trp}^{32}\text{-TNF}\alpha$ ; endotoxin content: 26 E.U./mg  
The last active fraction contained 1.8 mg/ml TNF and 47 E.U./mg protein.

#### 30 1. Anti-tumour effect of $\text{hTNF}\alpha$ and $\text{hIFN}\gamma$ on subcutaneous HT-29 tumours in nude mice.

5x10<sup>6</sup> HT-29 human colon adenocarcinoma cells [ATCC HTB38] were subcutaneously injected in nude mice. Groups consisted of 5 mice. The treatment comprises daily perilesional injections during 6 days per week, followed by 1 day without treatment. Results are given in Fig. 4 whereby "PBS" refers to phosphate buffered saline as known in the art. The single arrow indicates the start of the treatment with 5  $\mu\text{g}$   $\text{hTNF}\alpha$  or 5000 IU human Interferon  $\gamma$  ( $\text{hIFN}\gamma$ ) or both. The double arrow indicates the time that these doses were doubled and the crossed arrow indicates the end of the treatment.

#### 40 2. Comparison of the anti-tumour potential of $\text{hTNF}\alpha$ and $\text{Trp}^{32}\text{-TNF}\alpha$

5x10<sup>6</sup> HT-29 human colon adenocarcinoma cells (s.a.) were subcutaneously injected in nude mice. Groups consisted of 5 mice. The treatment started on day 6 following inoculation and comprises daily perilesional injections during 6 days per week. Tumour volume was estimated every 3 or 4 days by measuring the larger (a) and the smaller (b) diameter and calculating the  $a \times b^2 \times 0.4$  according to Attia and Weiss as known in the art. Results are given in Fig. 5 whereby the arrow indicates the start of the treatment and open triangles with tip down refers to 10<sup>4</sup> IU of  $\text{hIFN}\gamma$  and 10  $\mu\text{g}$   $\text{hTNF}\alpha$ , filled triangles with tip down refer to 10<sup>4</sup> IU of  $\text{hIFN}\gamma$  and 10  $\mu\text{g}$   $\text{Trp}^{32}\text{-TNF}$ , filled rectangles refer to 10  $\mu\text{g}$   $\text{Trp}^{32}\text{-TNF}\alpha$ , open rectangles refer to 10  $\mu\text{g}$   $\text{hTNF}\alpha$ , open triangles refer to phosphate buffered saline and filled circles refer to 10<sup>4</sup> IU of  $\text{hIFN}\gamma$ . In vitro, there is no difference in cytotoxicity for Hep or HT-29 cells between  $\text{hTNF}\alpha$  and 50  $\text{Trp}^{32}\text{-TNF}\alpha$ .

### Example V

#### Preparation of $\text{Ser}^{29}\text{-Trp}^{32}\text{-TNF}\alpha$

55  $\text{Ser}^{29}\text{-Trp}^{32}\text{-TNF}\alpha$  was prepared as described in Example I with the following exceptions:

1. The oligonucleotide used, contains the following sequence (mutated bases underlined):  
5'GGGCATTGGCCCGCGTTGACCACTGGAGC3'

2. An Nci 1 site was destroyed while an Ava 2-site was created, allowing for check of the presence of the mutation by restriction fragment analysis. No hybridization analysis was performed. 6 clones resulting from the WK6 transformation were grown up and DNA was prepared and analysed as described in Example I. 3 from 6 clones beared the mutation.

This DNA sequence was subcloned into the pDS56 expression vector, generating the plasmid pDS56/RBSII,Sph1-TNF $\alpha$ Ser29Trp32, and transformed to the E. coli M15 strain. Expression and purification was performed as described in Example I.

#### Example VI

##### Preparation of Gly<sup>29</sup>-TNF $\alpha$ , Tyr<sup>29</sup>-TNF $\alpha$ and Tyr<sup>32</sup>-TNF $\alpha$

Gly<sup>29</sup>-TNF $\alpha$ , Tyr<sup>29</sup>-TNF $\alpha$  and Tyr<sup>32</sup>-TNF $\alpha$  were prepared as described in Example I with the following exception. Oligonucleotides were used, containing a fully degenerated codon at position 29 or 32, resulting in a random insertion of all twenty amino acids at one of the two positions. The sequence of these oligonucleotides are as follows:

##### Position 29:

5' CCACGCCATTCGCGAGGAGGGCATTGGCCCGCGGTTXXXCCACTGGAGC 3'

##### Position 32:

5' CCACGCCATTCGCGAGGAGGGCATTGGCXXXGCGGTTTCAGCC 3'

where X = A, C, G or T and mutated bases are underlined.

Together with the mutation, also a unique Nru-1 site is introduced. Thus, instead of directly transforming the phasmid-pool, isolated from the WK6 mutS strain, this DNA was first digested with Nru-1, the linear band eluted from the agarose gel, ligated and transformed to the SURE-strain (Stratagene, La Jolla, CA, USA). In this way, one can select only for phasmids, containing the mutations. 168 colonies obtained were inoculated in microtiterplates, grown to confluency and their lysates tested for biological activity towards Hep-2 cells in a manner as described in Example IIa and for differential binding as described in Example IIb or Example VIII. On the basis of the biological activity on the one side and differential binding as determined according to Example IIb or Example VIII colonies were selected and further characterized by DNA sequence analysis of corresponding inserts as known in the art. DNA-sequences coding for Gly<sup>29</sup>-TNF $\alpha$ , Tyr<sup>29</sup>-TNF $\alpha$  and Tyr<sup>32</sup>-TNF $\alpha$  were isolated from corresponding colonies and cloned in bacterial expression vectors as described in Example I. Muteins expressed were purified to more than 95% homogeneity by means of a MONO-Q ion exchange chromatography step.

#### Example VII

##### Preparation of Glu<sup>31</sup>-TNF $\alpha$ and Asn<sup>31</sup>-Thr<sup>32</sup>-TNF $\alpha$

##### Mutagenesis of the TNF $\alpha$ gene using PCR

Three PCR reactions were performed with plasmid pDS56/RBSII,Sph1-TNF $\alpha$  [Figure 3] as the template DNA using a Perkin-Elmer Cetus GeneAmp™ DNA Amplification Reagent Kit with AmpliTaq™ Recombinant Taq DNA Polymerase (Perkin Elmer Cetus, Vaterstetten, BRD) [see Figure 8]. In reaction I primers 17/F (5'-GGCGTATCACGAGGCCCTTCG-3'; primer 17/F comprises nucleotides 3949-3970 of plasmid pDS56/RBSII,Sph1-TNF $\alpha$ ) and 21/M5 (5'-ATTGGCCCGCTCGTTCAGCCACTGGAGCTGCCCTC-3'; primer 21/M5 comprises nucleotides which are complementary to nucleotides 219-184 of plasmid pDS56/RBSII,Sph1-TNF $\alpha$ , mutated bases are underlined) were used, reaction II contained primers 17/F and 21/M6 (5'-ATTGGCAGTGTTGTTTCAGCCACTGGAGCTGCCCTC-3'; primer 21/M6 comprises nucleotides which are complementary to nucleotides 219-184 of plasmid pDS56/RBSII,Sph1-TNF $\alpha$ , mutated bases are underlined), and reaction III contained primers 21/MR (5'-GCCCTCCTGGCCAATGGCGTGG-3'; primer

21/MR comprises nucleotides 220-241 of plasmid pDS56/RBSII,Sph1-TNF $\alpha$  and 17/O (5'-CATTACTGGATCTATCAACAGG-3'; primer 17/O comprises nucleotides which are complementary to nucleotides 748-727 of plasmid pDS56/RBSII,Sph1-TNF $\alpha$ ). Therefore 10  $\mu$ l template DNA (10 ng), 5  $\mu$ l each of the two primers (100 pmole each), 16  $\mu$ l dNTP's mix (1.25 mM of dATP, dGTP, dCTP, and dTTP), 10  $\mu$ l 10x reaction buffer (100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM MgCl<sub>2</sub> and 0.1 % gelatin), 1  $\mu$ l (5 units) AmpliTaq™ DNA polymerase and 53  $\mu$ l H<sub>2</sub>O were mixed in an Eppendorf tube and overlaid with 80  $\mu$ l mineral oil (Perkin-Elmer Cetus). The tubes were transferred to a DNA thermal cycler (TRIO-Thermoblock, Biometra) and kept for 1 min at 94°C, before 35 cycles of melting the DNA (1 min at 94°C), annealing the primers (1 min at 50°C), and extending the primers (3 min at 72°C) were performed. After additional 2 min at 72°C, the reactions were cooled to room temperature and extracted with chloroform. The DNA present in the aqueous phase was precipitated with ethanol and subjected to electrophoresis in a 6 % polyacrylamide gel [Sambrook et al., 1989]. After staining of the DNA with ethidium bromide, fragments I, II and III [see Figure 8; these fragments originate from reactions I, II and III, respectively] were isolated from the gel and purified [Sambrook et al., 1989].

#### Preparation of DNA fragments encoding Glu<sup>31</sup>-TNF $\alpha$ and Asn<sup>31</sup>-Thr<sup>32</sup>-TNF $\alpha$

Fragments I, II and III were enzymatically phosphorylated, before in two parallel reactions fragments I and III and fragments II and III were ligated with each other [Sambrook et al., 1989]. After heat-inactivation of the ligase and digestion with restriction enzymes EcoRI and HindIII, the DNA was subjected to electrophoresis in a 6 % polyacrylamide gel. After staining of the DNA with ethidium bromide, the EcoRI-HindIII fragments A and B [see Figure 4] were isolated from the gel and purified [s.a].

#### Preparation of plasmids encoding Glu<sup>31</sup>-TNF $\alpha$ and Asn<sup>31</sup>-Thr<sup>32</sup>-TNF $\alpha$

In separate experiments, the EcoRI-HindIII fragments A and B were inserted according to standard methods [Sambrook et al., 1989] into the EcoRI-HindIII opened plasmid pDS56/RBSII,Sph1-TNF $\alpha$ Ser29 generating plasmids pDS56/RBSII,Sph1-TNF $\alpha$ Glu31 and pDS56/RBSII,Sph1-TNF $\alpha$ Asn31Thr32, respectively. Plasmid DNA was prepared [Birnboim et al., 1979] and the identity of the coding region for the TNF $\alpha$  mutants was confirmed by sequencing the double-stranded DNA [Sambrook et al., 1989].

#### Production of Glu<sup>31</sup>-TNF $\alpha$ and Asn<sup>31</sup>-Thr<sup>32</sup>-TNF $\alpha$

Plasmids pDS56/RBSII,Sph1-TNF $\alpha$ Glu31 and pDS56/RBSII,Sph1-TNF $\alpha$ Asn31Thr32 were transformed into E. coli M15 cells containing already plasmid pREP4 by standard methods [s.a.]. Transformed cells were grown at 37°C in LB medium [s.a.] containing 100 mg/l ampicillin and 25 mg/l kanamycin. At an optical density at 600 nm of about 0.7 to 1.0 IPTG was added to a final concentration of 2 mM. After additional 2.5 to 5 h at 37°C the cells were harvested by centrifugation.

#### Example VIII

##### Differential binding to recombinant human p75-TNF-R and recombinant human p55-TNF-R

1. 10 ml suspensions of transformed and induced E. coli cells expressing recombinant human TNF $\alpha$ , Ser<sup>29</sup>-TNF $\alpha$ , Trp<sup>32</sup>-TNF $\alpha$ , Glu<sup>31</sup>-TNF $\alpha$ , and Asn<sup>31</sup>-Thr<sup>32</sup>-TNF $\alpha$  [E. coli cells expressing recombinant dihydrofolate reductase (DHFR) were included as a control] were centrifuged at 4'000 rpm for 10 min and resuspended in 0.9 ml of lysis buffer (10 mM Tris-HCl pH 8.0, 5 mM EDTA, 2 mM PMSF, 10 mM benzamidine, 200 units/ml aprotinin and 0.1 mg/ml lysozyme). After 20 min incubation at room temperature 50  $\mu$ l of 1 M MgCl<sub>2</sub>, 20  $\mu$ l of 5 mg/ml DNaseI, 50  $\mu$ l of 5 M NaCl and 50  $\mu$ l of 10% NP-40 were added and the mixture was further incubated at room temperature for 15 min. 0.5 ml of the lysate clarified by centrifugation at 13'000 rpm for 5 min was subjected to ammonium sulfate precipitation (30% - 70% cut). The 70% ammonium sulfate pellet was dissolved in 0.2 ml PBS and analyzed by SDS-PAGE to confirm the presence of the recombinant proteins.

For the differential binding assay microtiter plates were coated with recombinant human p75-TNF-R-human IgG $\gamma$ 3 and p55-TNF-R-human IgG $\gamma$ 3 fusion proteins (European Patent Applications Publ. Nos. 417 563, 422 339) dissolved in PBS at 0.3  $\mu$ g/ml and 0.1  $\mu$ g/ml, respectively, (100  $\mu$ l/well, overnight at 4°C). After blocking with blocking buffer (50 mM Tris pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% NaN<sub>3</sub>, 1% defatted milk powder) the microtiter plate was washed with PBS and incubated with 5 ng/ml human

<sup>125</sup>I-TNF $\alpha$  (labelled by the lodogen method to a specific activity of about 30  $\mu$ Ci/ $\mu$ g as described above) in the presence of different dilutions of the E. coli lysate partially purified by ammonium sulfate precipitation. The volume was 100  $\mu$ l/well and each dilution was assayed in duplicate. After three hours at room temperature the wells were thoroughly washed with PBS and counted in a  $\gamma$ -counter. Results are shown in Fig.6 whereby closed circles refer to binding to p55-TNF-R-human IgG $\gamma$ 3- and open circles refer to binding to p75-TNF-R-human IgG $\gamma$ 3.

2. Determination of binding of Ser<sup>29</sup>-Trp<sup>32</sup>-TNF $\alpha$ , Gly<sup>29</sup>-TNF $\alpha$ , Tyr<sup>29</sup>-TNF $\alpha$  and Tyr<sup>32</sup>-TNF $\alpha$  was performed as described under 1. with the only exception that MONO-Q ion exchange chromatography purified muteins were used. Results are shown in Fig. 7 whereby open and closed circles have the same meaning as in Fig. 6 and  $\mu$ g/ml gives the amount of purified mutein/ml.

#### Claims

1. A human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor.

2. A mutein as claimed in claim 1 or a pharmaceutically acceptable salt thereof wherein the amino acid sequence of human Tumor Necrosis Factor is

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1                               10
VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS
25                               20                               30
VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN
                               40
ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP
30                               50                               60
ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER
                               70
GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU
35                               80                               90
LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS
40                               100
VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR
                               110                               120
PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU
45                               130
GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU
                               140                               150
ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL
50                               157
TYR PHE GLY ILE ILE ALA LEU.
55

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3. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed by substitution of one or more, preferably one or two amino acids by other amino



acids, preferably naturally occurring amino acids.

4. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at position 29 as claimed in claim 3.
- 5 5. A mutein as claimed in claim 4 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is serine.
6. A mutein as claimed in claim 4 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is glycine.
- 10 7. A mutein as claimed in claim 4 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is tyrosine.
- 15 8. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at position 32 as claimed in claim 3.
9. A mutein as claimed in claim 8 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is tryptophan.
- 20 10. A mutein as claimed in claim 8 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is tyrosine.
11. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at position 31 as claimed in claim 3.
- 25 12. A mutein as claimed in claim 11 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid is glutamic acid.
- 30 13. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at positions 29 and 32 as claimed in claim 3.
14. A mutein as claimed in claim 13 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid at position 29 is serine, glycine or tyrosine, preferably serine and at position 32 tyrosine or tryptophan, preferably tryptophan.
- 35 15. A mutein as claimed in claim 2 or a pharmaceutically acceptable salt thereof wherein said amino acid sequence is changed at positions 31 and 32.
- 40 16. A mutein as claimed in claim 15 or a pharmaceutically acceptable salt thereof wherein said naturally occurring amino acid at position 31 is glutamic acid or asparagine, preferably asparagine and at position 32 tyrosine, tryptophan or threonine, preferably threonine.
- 45 17. A deletion, substitution and/or addition analog of a mutein as claimed in any one of claims 4-16 or a pharmaceutically acceptable salt thereof whereby position 29 and/or 32 or position 31 or position 31 and 32 of the mutein is/are not changed and which analog still shows a significant difference between its binding affinity to the human p75-Tumor Necrosis-Factor-Receptor and the human p55-Tumor Necrosis-Factor-Receptor.
- 50 18. A DNA-sequence comprising a DNA-sequence coding for a mutein as claimed in any one of claims 1-17.
19. A vector, especially for expression in a procaryotic or lower eukaryotic host cell, such vector comprising a DNA-sequence as claimed in claim 18.
- 55 20. A host cell, especially a procaryotic or lower eukaryotic host cell transformed by a vector as claimed in claim 19.

21. A host cell as claimed in claim 20 which is E. coli.

22. A compound as claimed in any one of claims 1-17 for the treatment of illnesses.

5 23. A process for the preparation of a compound as claimed in any one of claims 1-17 which process comprises cultivating a host cell as claimed in claim 20 or claim 21 in a suitable medium and isolating the mutein from the culture supernatant or the host cell itself, and if desired converting said mutein into a pharmaceutically acceptable salt.

10 24. A pharmaceutical composition which contains one or more compounds as claimed in any one of claims 1-17, if desired, in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

25. The use of a compound as claimed in any one of claims 1-17 for the treatment of illnesses.

15

**Claims for the following Contracting States : GR,ES**

1. A process for the preparation of a human Tumor Necrosis Factor mutein or a pharmaceutically acceptable salt thereof which mutein is characterized in that the TNF sequence is changed by deletion, insertion and/or substitution of one or more amino acids so that the mutein shows a significant difference between its binding affinity to the human p75-Tumor-Necrosis-Factor-Receptor and to the human p55-Tumor-Necrosis-Factor-Receptor which process comprises cultivating a host cell transformed with an expression vector comprising a DNA sequence coding for such a mutein in a suitable medium and isolating this mutein from the culture supernatant or the host cell itself, and if desired converting this mutein into a pharmaceutically acceptable salt.

25

2. A process as claimed in claim 1 whereby the amino acid sequence of human Tumor Necrosis Factor is

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1          10
30  VAL ARG SER SER SER ARG THR PRO SER ASP LYS PRO VAL ALA HIS

          20          30
    VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN

35          40
    ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY VAL GLU LEU ARG ASP

          50          60
40  ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER

          70
    GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO SER THR HIS VAL LEU

          80          90
45  LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS

          100
    VAL ASN LEU LEU SER ALA ILE LYS SER PRO CYS GLN ARG GLU THR

50          110          120
    PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU

          130
55  GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP ARG LEU SER ALA GLU

```

140  
 ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL 150  
 157  
 5 TYR PHE GLY ILE ILE ALA LEU.

3. A process as claimed in claim 2 whereby said amino acid sequence is changed by substitution of one or more, preferably one or two amino acids by other amino acids, preferably naturally occurring amino acids.  
10
4. A process as claimed in claim 2 whereby said amino acid sequence is changed at position 29 as claimed in claim 3.  
15
5. A process as claimed in claim 4 whereby said naturally occurring amino acid is serine.
6. A process as claimed in claim 4 whereby said naturally occurring amino acid is glycine.
- 20 7. A process as claimed in claim 4 whereby said naturally occurring amino acid is tyrosine.
8. A process as claimed in claim 2 whereby said amino acid sequence is changed at position 32 as claimed in claim 3.
- 25 9. A process as claimed in claim 8 whereby said naturally occurring amino acid is tryptophan.
10. A process as claimed in claim 8 whereby said naturally occurring amino acid is tyrosine.
11. A process as claimed in claim 2 whereby said amino acid sequence is changed at position 31 as claimed in claim 3.  
30
12. A process as claimed in claim 11 whereby said naturally occurring amino acid is glutamic acid.
13. A process as claimed in claim 2 whereby said amino acid sequence is changed at positions 29 and 32 as claimed in claim 3.  
35
14. A process as claimed in claim 13 whereby said naturally occurring amino acid at position 29 is serine, glycine or tyrosine, preferably serine and at position 32 tyrosine or tryptophane, preferably tryptophan.
- 40 15. A process as claimed in claim 2 whereby said amino acid sequence is changed at positions 31 and 32.
16. A process as claimed in claim 15 whereby said naturally occurring amino acid at position 31 is glutamic acid or asparagin, preferably asparagine and at position 32 tyrosine, tryptophan or threonine, preferably threonine.  
45
17. A process for the preparation of a deletion, substitution and/or addition analog of a mutein obtained by a process as claimed in any one of claims 4-16 or a pharmaceutically acceptable salt thereof whereby position 29 and/or 32 or position 31 or position 31 and 32 of said mutein is/are not changed and which analog still shows a significant difference between its binding affinity to the human p75-Tumor Necrosis-Factor-Receptor and the human p55-Tumor Necrosis-Factor-Receptor which process comprises cultivating a host cell transformed with an expression vector comprising a DNA sequence coding for such an analog in a suitable medium and isolating the analog from the culture supernatant or the host cell itself, and if desired converting said analog into a pharmaceutically acceptable salt.  
50
18. A process as claimed in any one of claims 1-17 whereby the host cell is a procaryotic or lower eukaryotic host cell.  
55
19. A process as claimed in claim 18 whereby the prokaryotic host cell is E. coli.

20. A process as claimed in any one of claims 1-19 whereby the expression vector is a vector of the pDS family.
- 5 21. A process for the preparation of a pharmaceutical composition which process is characterized in that a compound obtained by a process as claimed in any one of claims 1-20 and if desired, additional pharmaceutically active substances are mixed with a non-toxic, inert, therapeutically compatible carrier material and the mixture is brought into a galenical application form.
- 10 22. A pharmaceutical composition which contains one or more compounds obtained according to a process as claimed in any one of claims 1-20, if desired, in combination with additional pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.
- 15 23. The use of a compound prepared according to a process as claimed in any one of claims 1-20 for the preparation of a pharmaceutical composition according to claim 22.
24. A compound whenever prepared according to a process as claimed in any one of claims 1-20.
25. The invention as hereinbefore described.
- 20 26. A DNA-sequence comprising a DNA-sequence coding for a compound prepared according to a process as claimed in any one of claims 1-20.
27. A vector, especially for expression in a procaryotic or lower eukaryotic host cell, such vector comprising a DNA-sequence as claimed in claim 26.
- 25 28. A host cell, especially a procaryotic or lower eukaryotic host cell transformed by a vector as claimed in claim 27.
- 30 29. A host cell as claimed in claim 28 which is E. coli.

Fig. 1a

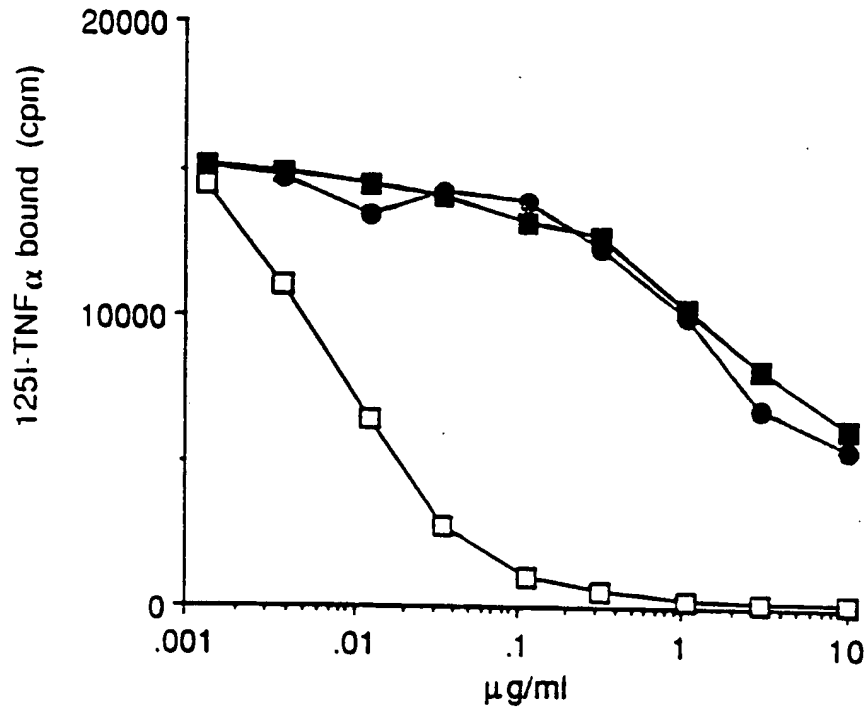


Fig. 1b

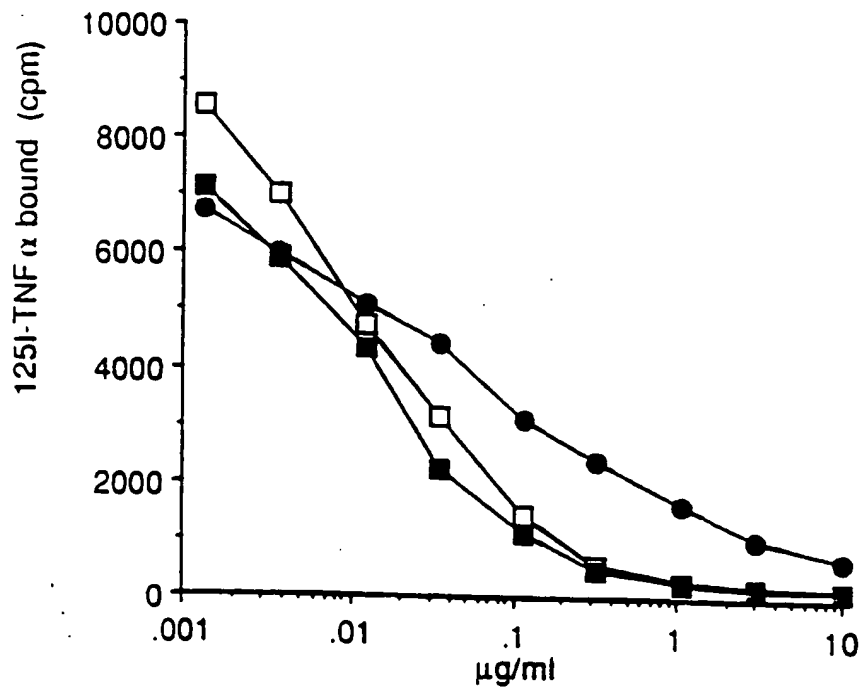


Fig. 2a

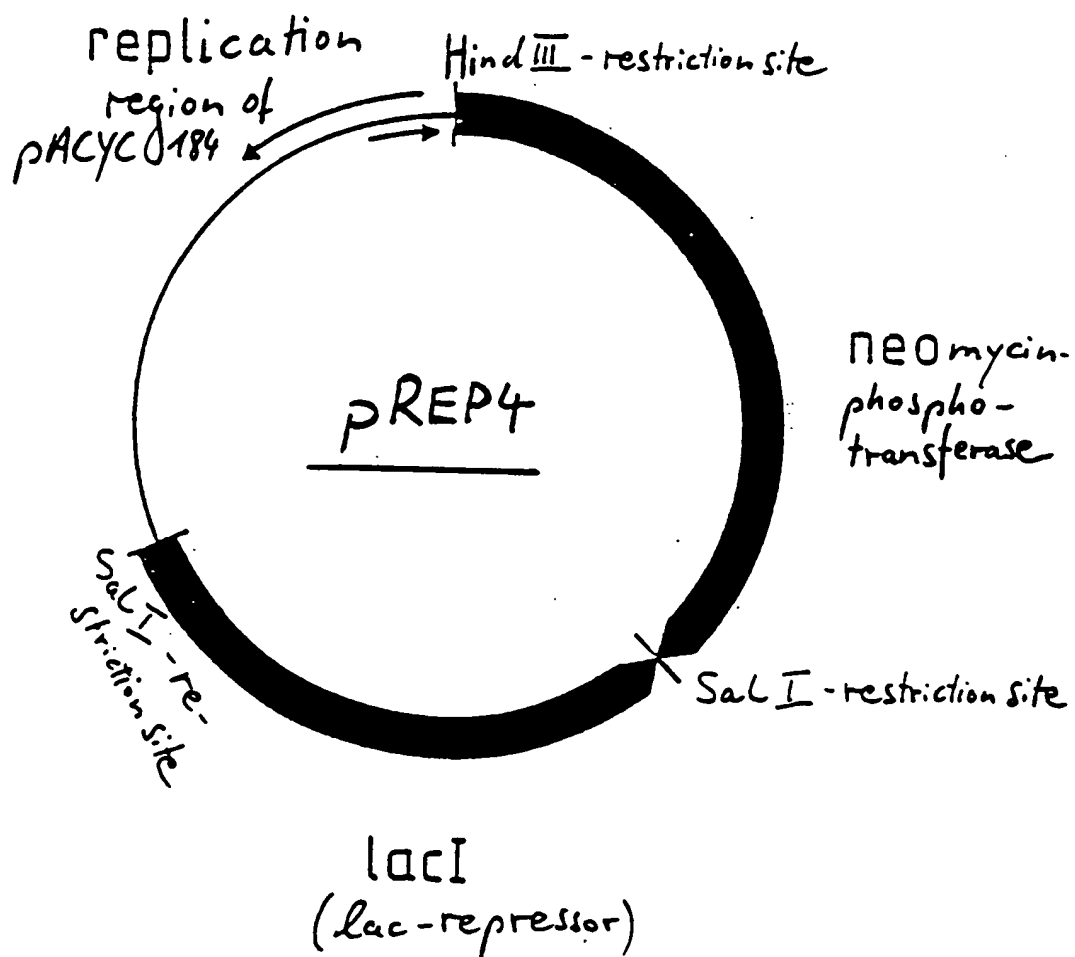


Fig. 2b/1

HindIII

1 AAGCTTCACG CTGCCGCAAG CACTCAGGGC GCAAGGGCTG CTAAGGAAG  
51 CGGAACACGT AGAAAGCCAG TCCGAGAAA CGGTGCTGAC CCCGGATGAA  
101 TGTCAGCTAC TGGGCTATCT GGACAAGGGA AAACGCAAGC GCAAAGAGAA  
151 AGCAGGTAGC TTGCAGTGGG CTTACATGGC GATAGCTAGA CTGGGCGGTT  
201 TTATGGACAG CAAGCGAACC GGAATTGCCA GCTGGGGGCG CCTCTGGTAA  
251 GGTGGGAAG CCCTGCAAAG TAAACTGGAT GGCTTTCTTG CCGCCAAGGA  
301 TCTGATGGCG CAGGGGATCA AGATCTGATC AAGAGACAGG ATGACGGTCG  
351 TTTCCGATGC TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCCGCTTG  
401 GGTGGAGAGG CTATTGGGCT ATGACTGGGC ACAACAGACA ATCGGCTGCT  
451 CTGATGCCGC CGTGTTCCGG CTGTCAGCGC AGGGGGCGCC GGTCTTTTTT  
501 GTCAAGACCG ACCTGTCCGG TGCCCTGAAT GAACTGCAGG ACGAGGCAGC  
551 GCGGCTATCG TGGCTGGCCA CGACGGGCGT TCCTTGCGCA GCTGTGCTCG  
601 ACGTTGTAC TGAAGCGGA AGGGACTGGC TGCTATTGGG CGAAGTGCCG  
651 GGGCAGGATC TCCTGTCAAT TCACCTTGCT CCTGCCGAGA AAGTATCCAT  
701 CATGGCTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC  
751 CATTGACCA CCAAGCGAAA CATCGCATCG AGCGAGCAGG TACTCGGATG  
801 GAAGCCGGTC TTGTGATCA GGATGATCTG GAAGAAGAGC ATCAGGGGCT  
851 CCGCCAGCC GAACTGTTTG CCAGGCTCAA GGCGCGCATG CCCGACGGCG  
901 AGGATCTCGT CGTGACCCAT GGCGATGCCT GCTTGCGAA TATCATGGTG  
951 GAAATGGCC GCTTTTCTGG ATTCAATGAC TGTGGCCGCG TGGGTGTGGC  
1001 GGACCGCTAT CAGGACATAG CGTTGGCTAC CCGTGATATT GCTGAAGAGC  
1051 TTGGCGGCGA ATGGGCTGAC CGCTTCCTCG TGCTTTACGG TATCGCCGCT  
1101 CCCGATTCGC AGCGCATCGC CTTCATATCG CTTCCTGACG AGTTCTTCTG  
1151 AGCGGGACTC TGGGGTTCGA AATGACCGAC CAAGCGACGC CCAACCTGCC  
1201 ATCAGGAGAT TTCGATTCCA CGCGCGCTT CTATGAAAGG TTGGGCTTCG  
1251 GAATCGTTTT CCGGACGCC GGCTGGATGA TCCTCCAGCG CGGGGATCTC  
1301 ATGCTGGAGT TCTTCGCCA CCCCGGGCTC GATCCCTCG CGAGTTGGTT

Fig. 2b/2

1351 CAGCTGCTGC CTGAGGCTGG ACGACCTCGC GGAGTTCTAC CGGCAGTGCA  
 1401 AATCGGTCGG CATCCAGGAA ACCAGCAGCG GCTATCCGCG CATCCATGCC  
 1451 CCGGAAGTGC AGGAGTGGGG AGGCACGATG GCGGCTTTGG TCGACAATTC  
 1501 GCGCTAACTT ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG  
 1551 GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA  
 1601 GCGGGTTTGC GTATTGGGCG CCAGGGTGGT TTTTCTTTTC ACCAGTGAGA  
 1651 CGGCAACAG CTGATTGCCC TTCACCGCCT GGCCCTGAGA GAGTTGCAGC  
 1701 AAGCGGTCCA CGCTGGTTTG CCCAGCAGG CGAAATCCT GTTGATGGT  
 1751 GGTTAACGGC GGGATATAAC ATGAGCTGTC TTCGGTATCG TCGTATCCCA  
 1801 CTACCGAGAT ATCCGCACCA ACGCGCAGCC CGGACTCGGT AATGGCGCGC  
 1851 ATTGGCCCCA GCGCCATCTG ATCGTTGGCA ACCAGCATCG CAGTGGGAAC  
 1901 GATGCCCTCA TTCAGCATTT GCATGGTTTG TTGAAAACCG GACATGGCAC  
 1951 TCCAGTCGCC TTCCCGTTCC GCTATCGGCT GAATTTGATT GCGAGTGAGA  
 2001 TATTTATGCC AGCCAGCCAG ACGCAGACGC GCGGAGACAG AACTTAATGG  
 2051 GCGCGCTAAC AGCGCGATTT GCTGGTGACC CAATGCGACC AGATGCTCCA  
 2101 CGCCAGTCG CGTACCGTCT TCATGGGAGA AAATAATACT GTTGATGGGT  
 2151 GTCTGGTCAG AGACATCAAG AAATAACGCC GGAACATTAG TGCAGGCAGC  
 2201 TTCCACAGCA ATGGCATCCT GGTCAATCCAG CGGATAGTTA ATGATCAGCC  
 2251 CACTGACGCG TTGCGCGAGA AGATTGTGCA CCGCCGCTTT ACAGGCTTCG  
 2301 ACGCCGCTTC GTTCTACCAT CGACACCACC ACGCTGGCAC CCAGTTGATC  
 2351 GGCGCGAGAT TTAATCGCGG CGACAATTTG CGACGGCGCG TGCAGGGCCA  
 2401 GACTGGAGGT GGCAACGCCA ATCAGCAACG ACTGTTTGCC CGCCAGTTGT  
 2451 TGTGCCACGC GGTGGGAAT GTAATTCAGC TCGGCCATCG CCGCTTCCAC  
 2501 TTTTCCCGC GTTTTCGCAG AAACGTGGCT GGCTGGTTC ACCACGCGGG  
 2551 AAACGGTCTG ATAAGAGACA CCGGCATACT CTGCGACATC GTATAAGTT  
 2601 ACTGGTTTCA CATTCACCAC CCTGAATTGA CTCTCTTCG GCGCTATCA  
 2651 TGCCATACCG CGAAAGGTTT TCGCCATTC GATGGTGTCA ACGTAAATGC  
 2701 ATGCCGCTTC GCCTTCGCGC GCGAATTTG GACCTGTCC CTCCTGTCA



Fig. 2b/3

2751 GCTACTGACG GGTGGTGCG TAACGGCAAA AGCACCGCCG GACATCAGCG  
 2801 CTAGCGGAGT GTATACTGGC TTACTATGTT GGCCTGATG AGGGTGTGAG  
 2851 TGAAGTGCTT CATGTGGCAG GAGAAAAAAG GCTGCACCGG TCGTTCAGCA  
 2901 GAATATGTGA TACAGGATAT ATTCCGCTTC CTCGCTCACT GACTCGCTAC  
 2951 GCTCGGTCGT TCGACTGCGG CGAGCGGAAA TGGCTTACGA ACGGGGCGGA  
 3001 GATTTCCTGG AAGATGCCAG GAAGATACTT AACAGGGAAG TGAGAGGGCC  
 3051 GCGGCAAAGC CGTTTTTCCA TAGGCTCCGC CCCCCTGACA AGCATCACGA  
 3101 AATCTGACGC TCAAATCAGT GGTGGCGAAA CCGACAGGA CTATAAAGAT  
 3151 ACCAGGCGTT TCCCCTGGCG GCTCCCTCGT GCGCTCTCCT GTTCTGCTT  
 3201 TTGGTTTAC CGGTGTCATT CCGCTGTTAT GGCCGCGTTT GTCTCATTC  
 3251 ACGCCTGACA CTCAGTTCG GGTAGGCAGT TCGCTCCAAG CTGGACTGTA  
 3301 TGCACGAACC CCGGTTTCAG TCCGACCGCT GCGCCTTATC CGGTAACAT  
 3351 CGTCTTGAGT CCAACCCGGA AAGACATGCA AAAGCACCAC TGGCAGCAGC  
 3401 CACTGGTAAT TGATTAGAG GAGTTAGTCT TGAAGTCATG CGCCGGTTAA  
 3451 GGCTAAACTG AAAGGACAAG TTTTGGTGAC TCGCTCCTC CAAGCCAGTT  
 3501 ACCTCGGTTT AAAGAGTTGG TAGCTCAGAG AACCTTCGAA AAACCGCCCT  
 3551 GCAAGGCGGT TTTTTCGTTT TCAGAGCAAG AGATTACGGG CAGACCAAAA  
 3601 CGATCTCAAG AAGATCATCT TATTAATCAG ATAAAATATT TCTAGATTTT  
 3651 AGTGCAATTT ATCTCTTCAA ATGTAGCACC TGAAGTCAGC CCCATACGAT  
 3701 ATAAGTTGTT AATTCTCATG TTTGACAGCT TATCATCGAT

Fig. 3a

N250PSN250P29

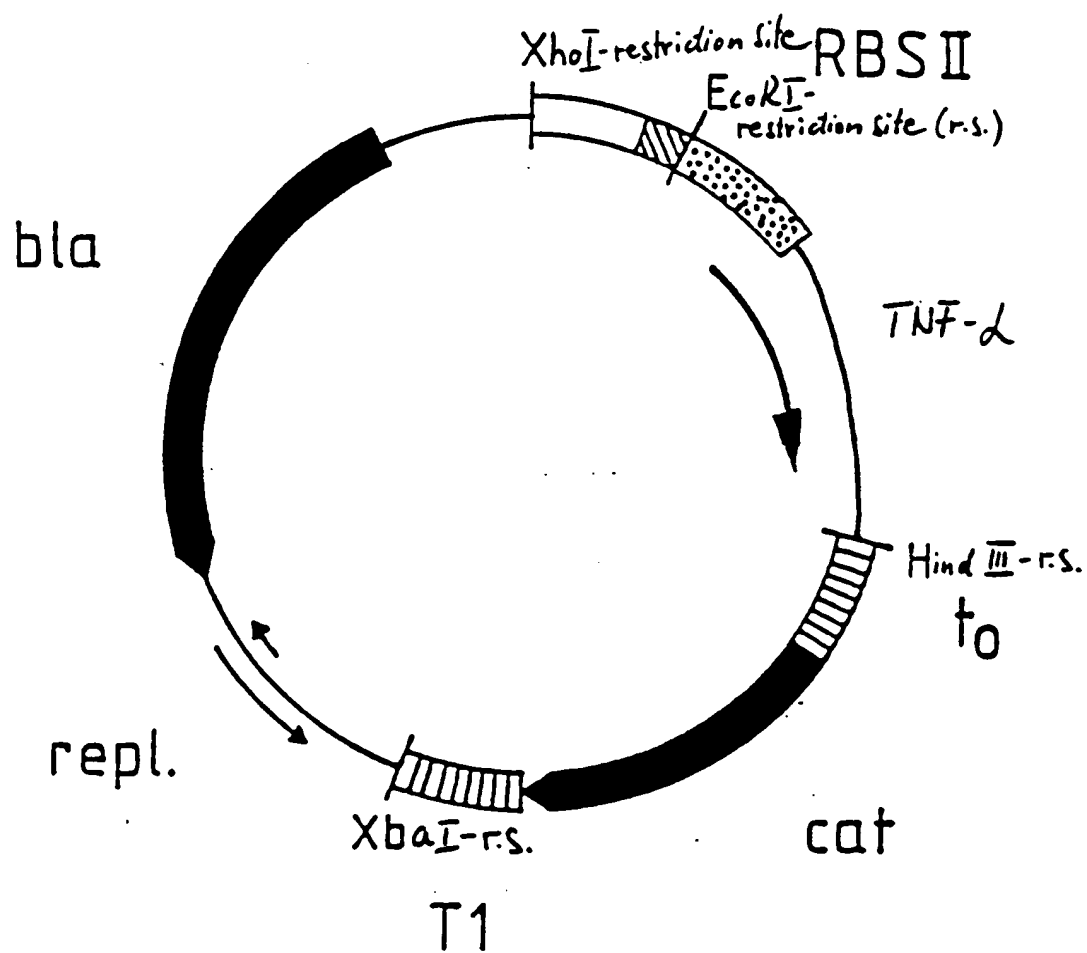


Fig. 3b/1

Xho I  
 1 CTGAGAAAT CATAAAAAAT TTATTGCTT TGTGAGCGGA TAACAATTAT  
 51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCAATTAAAG  
 101 AGGAGAAATT AAGCATGGTC AGATCATCTT CTGGAACCCC GAGTGACAAG  
 151 CCTGTAGCCC ATGTTGTGCG GAACCTCAA GCTGAGGGGC AGCTCCAGTG  
 201 GCTGAACCGC CGGGCCAATG CCCTCCTGGC CAATGGCGTG GAGCTGAGAG  
 251 ATAACCAGCT GGTGGTGCCA TCAGAGGGCC TGTACCTCAT CTACTCCCAG  
 301 GTCTCTTCA AGGGCCAAGG CTGCCCCCTC ACCCATGTGC TCCTCACCCA  
 351 CACCATCAGC CGCATCGCCG TCTCCTACCA GACCAAGGTC AACCTCCTCT  
 401 CTGCCATCAA GAGCCCCTGC CAGAGGGAGA CCCAGAGGG GGCTGAGGCC  
 451 AAGCCCTGGT ATGAGCCCAT CTATCTGGGA GGGGTCTTCC AGCTGGAGAA  
 501 GGGTGACCGA CTCAGCGCTG AGATCAATCG GCCCCACTAT CTGACTTTG  
 551 CCGAGTCTGG GCAGGTCTAC TTTGGGATCA TTGCCCTGTG AGGAGGAOGA  
 601 ACATCCAACC TTCCCAAAGC CCTCCCCTGC CCCAATCCCT TTATTACCCC  
 651 CTCCTTCAGA CACCCTCAAC CTCTTCTGGC TCAAAAAGAG AATTGGGGGC  
 701 TTAGGGTCCG AACCCAGCT TGGACTCCTG TTGATAGATC CAGTAATGAC  
 751 CTCAGAACTC CATCTGGATT TGTCAGAAC GCTCGGTTGC CGCCGGGCGT  
 801 TTTTATTGG TGAGAATCCA AGCTAGCTTG GCGAGATTTT CAGGAGCTAA  
 851 GGAAGCTAAA ATGGAGAAAA AAATCACTGG ATATACCACC GTTGATATAT  
 901 CCCAATGGCA TCGTAAAGAA CATTTTGAGG CATTTTCAGTC AGTTGCTCAA  
 951 TGTACCTATA ACCAGACCGT TCAGCTGGAT ATTACGGCCT TTTTAAAGAC  
 1001 CGTAAAGAAA AATAAGCACA AGTTTATCC GGCCTTTATT CACATTCTTG  
 1051 CCCGCCGTAT GAATGCTCAT CCGGAATTTT GTATGGCAAT GAAAGACGGT  
 1101 GAGCTGGTGA TATGGGATAG TGTCACCCT TGTTACACCG TTTTCCATGA  
 1151 GCAAAC TGAA ACGTTTTCAT CGCTCTGGAG TGAATACCAC GACGATTTCC  
 1201 GGCAGTTTCT ACACATATAT TCGCAAGATG TGGCGTGTTA CCGTGAAAAC  
 1251 CTGGCCTATT TCCCTAAAGG GTTTATGAG AATATGTTTT TCGTCTCAGC

Fig. 3b/2

1301 CAATCCCTGG GTGAGTTTCA CCAGTTTGA TTAAACGTG GCCAATATGG  
 1351 ACAACTTCTT CGCCCCGTT TTCACCATGG GCAATATTA TAAGCAAGGC  
 1401 GACAAGGTGC TGATGCCGCT GCGATTGAG GTTCATCATG CCGTCTGTGA  
 1451 TGGCTTCCAT GTGGGCAGAA TGCTTAATGA ATTACAACAG TACTGCCGATG  
 1501 AGTGGCAGGG CGGGGCGTAA TTTTITTAAG GCAGTATTG GTGCCCTTAA  
 1551 ACGCCTGGGG TAATGACTCT CTAGCTTGAG GCATCAAATA AAACGAAAGG  
 1601 CTCAGTGGAA AGACTGGGCC TTCTGTTTGA TCTGTGTTT GTGGGTGAAC  
 1651 GCTCTCCTGA GTAGGACAAA TCCGCGCTC TAGAGCTGCC TCGCGCGTTT  
 1701 CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA  
 1751 CAGCTTGTCT GTAAGCGGAT GCGGGAGCA GACAAGCCCG TCAGGGCGCG  
 1801 TCAGCGGGTG TTGGCGGGTG TCGGGCGCA GCCATGACCC AGTCACGTAG  
 1851 CGATAGCGGA GTGTATACTG GCTTAATAT GCGGCATCAG AGCAGATTGT  
 1901 ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA TCGTAAGGA  
 1951 GAAAATACCG CATCAGGCGC TCTCCGCTT CCTCGCTCAC TGAATCGCTG  
 2001 CGCTCGGTCT GTGGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT  
 2051 AATACGGTTA TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG  
 2101 CAAAAGGCCA GCAAAGGCC AGGAACCGTA AAAAGGCCGC GTTGCTGGCG  
 2151 TTTTCCATA GGCTCCGCCC CCCTGACGAG CATCAGAAA ATCGACGCTC  
 2201 AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC  
 2251 CCCCTGGAAG CTCCCTCGTG CGCTCTCTG TTCCGACCTT GCGCTTACC  
 2301 GGATACCTGT CGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCAATG  
 2351 CTCACGCTGT AGGTATCTCA GTTCGGTGTA GGTGTTTCCG TCCAAGCTGG  
 2401 GCTGTGTGCA CGAACCCCC GTTCAGCCCG ACCGCTGGCG CTATCCGGT  
 2451 AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT CGCCACTGGC  
 2501 AGCAGCCACT GGTACAGGA TTAGCAGAGC GAGGTATGTA GCGGGTGCTA  
 2551 CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA  
 2601 TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG  
 2651 TAGCTCTTGA TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTITTTTTG

Fig. 3b/3

2701 TTTGCAAGCA GCAGATTACG CGCAGAAAAA AAGGATCTCA AGAAGATCCT  
 2751 TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA ACTCACGTTA  
 2801 AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT  
 2851 TAAATTAATA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT  
 2901 TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT  
 2951 CTGTCTATTT CGTTCATCCA TAGCTGCCTG ACTCCCCGTC GTGTAGATAA  
 3001 CTACGATACG GGAGGGCTTA CCATCTGGCC CCAGTGCTGC AATGATACCG  
 3051 CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA ACCAGCCAGC  
 3101 CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC  
 3151 AGTCTATTAA TTGTGCGCG GAAGCTAGAG TAAGTAGTTC GCCAGTAAAT  
 3201 AGTTTGCGCA ACGTGTGTGC CATGTCTACA GGCATCGTGG TGTACGCTC  
 3251 GTCGTTTGGT ATGGCTTCAT TCAGCTCOGG TTCCCAACGA TCAAGGCGAG  
 3301 TTACATGATC CCCCATGTTG TGCAAAAAG CGGTTAGCTC CTTGGTCTCT  
 3351 CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTATCAC TCATGGTTAT  
 3401 GGCAGCACTG CATAATTCTC TTAATGTCTG GCCATCCGTA AGATGCTTTT  
 3451 CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCCG  
 3501 CGACCGAGTT GCTCTTGCCC GCGGTCAATA CGGGATAATA CCGCGCCACA  
 3551 TAGCAGAACT TAAAAAGTGC TCATCATTGG AAAACGTTCT TCGGGGCGAA  
 3601 AACTCTCAAG GATCTTACCG CTGTTGAGAT CCAGTTCGAT GTAACCCACT  
 3651 CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA GCGTTTCTGG  
 3701 GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA  
 3751 CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCATA TTATTGAAGC  
 3801 ATTATCAGG GTTATGTCT CATGAGCGGA TACATATTG AATGTATTTA  
 3851 GAAAAATAAA CAAATAGGGG TTCCGCGCAC ATTTCCCGA AAAGTGCCAC  
 3901 CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA TAAAAATAGG  
 3951 CGTATCACGA GGCCCTTTTC TCTTCAC

Fig. 4

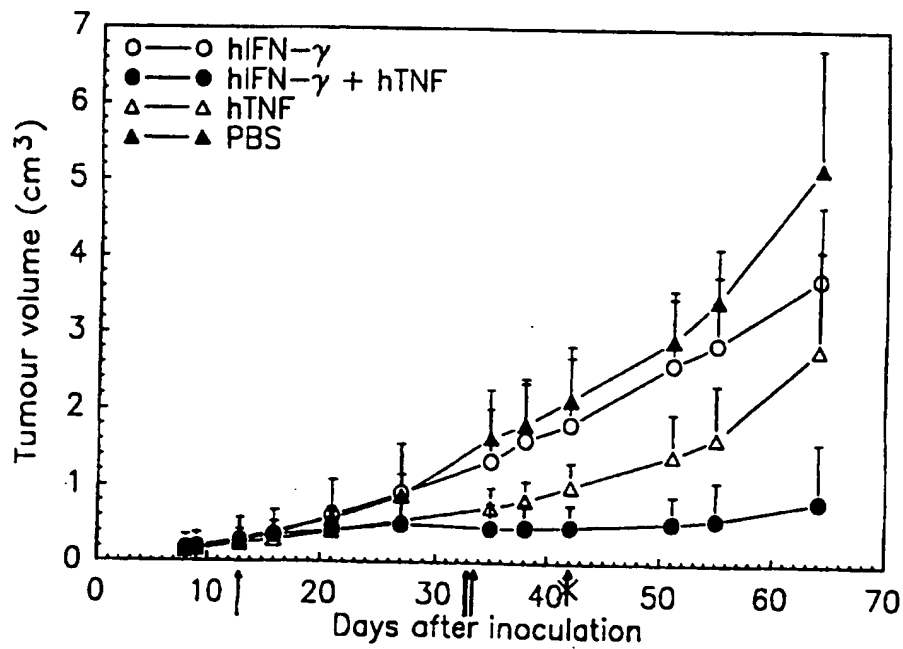


Fig. 5

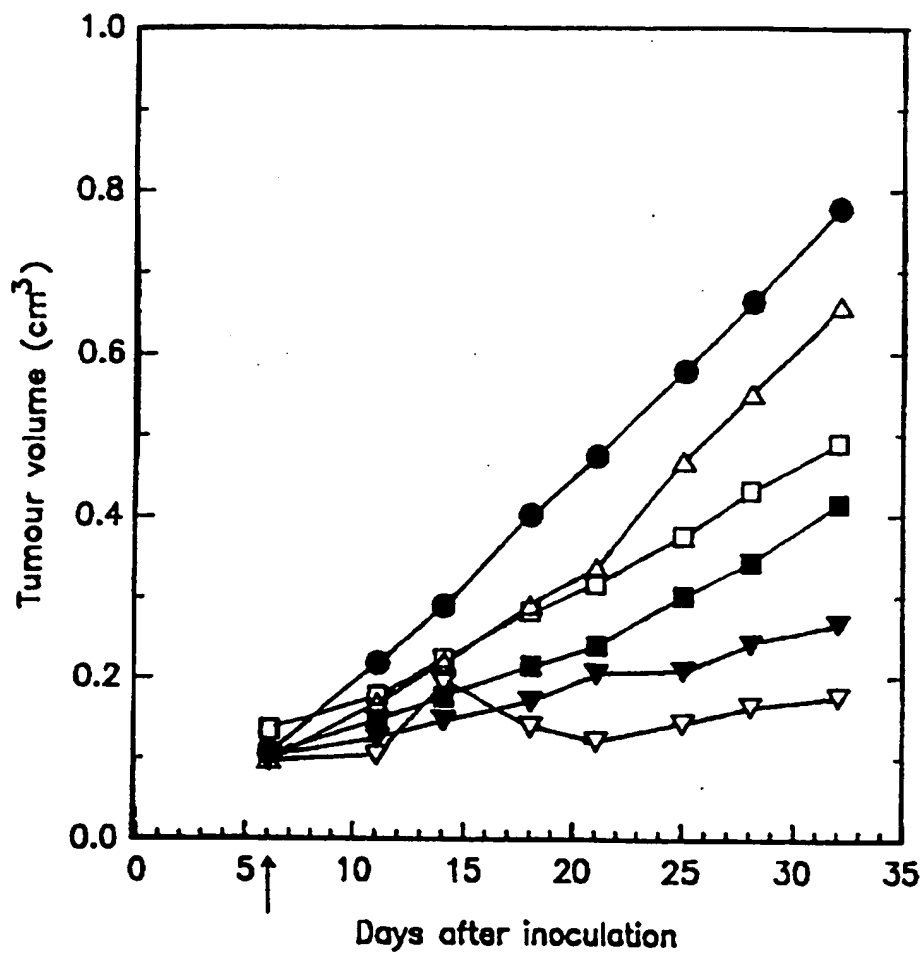
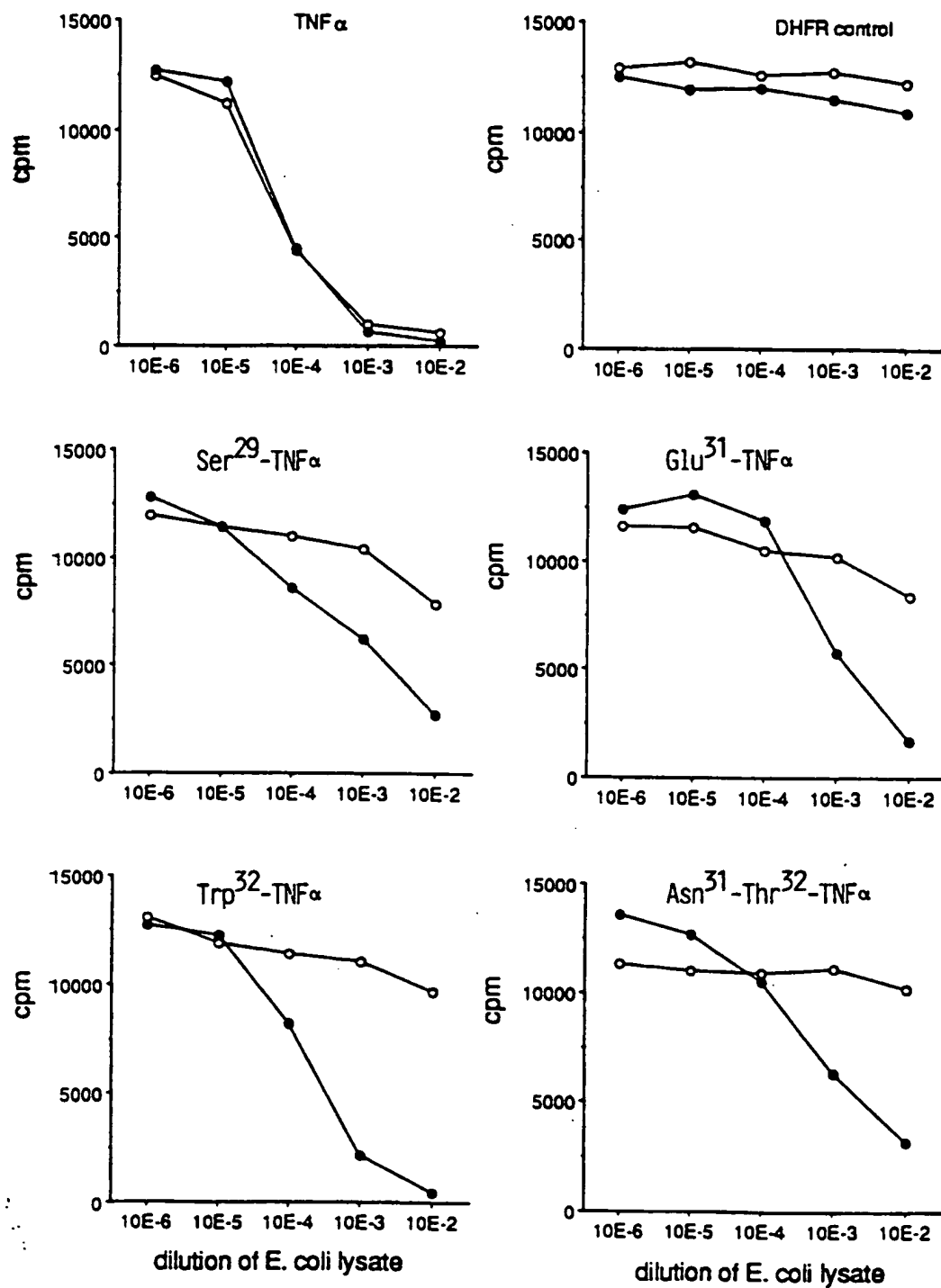


Fig. 6





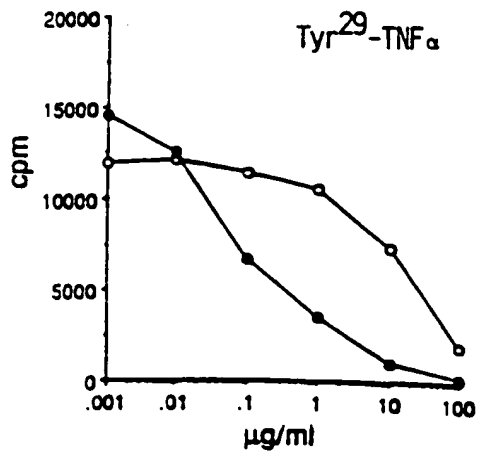
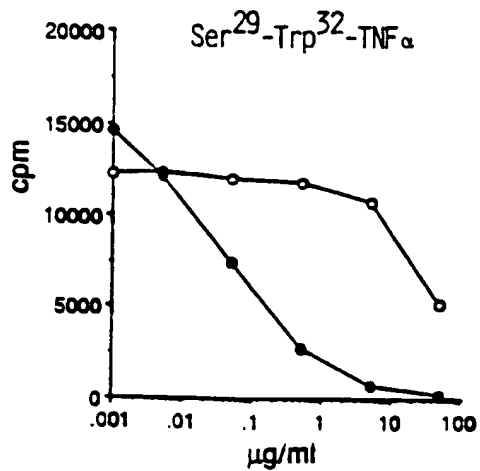
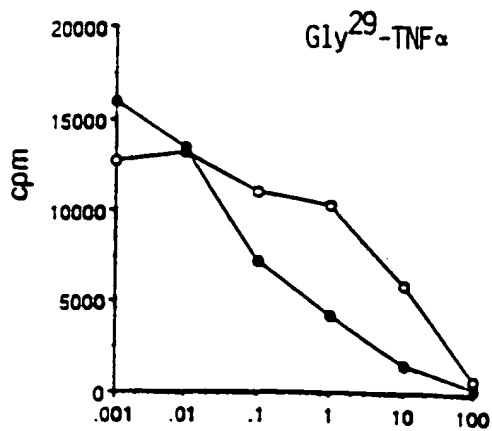
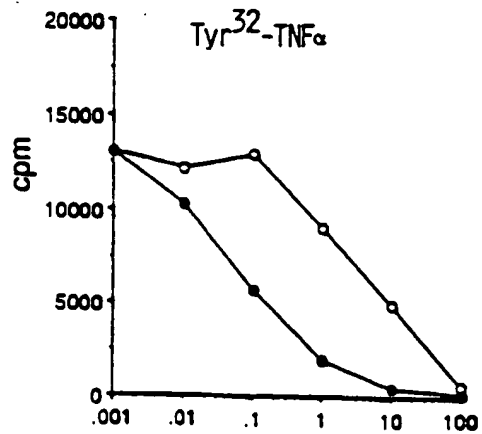
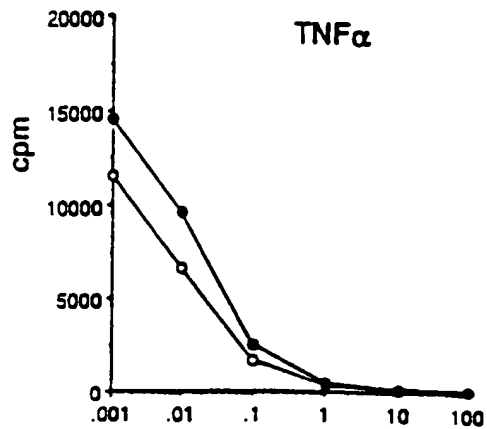
**Fig. 7**

Fig. 8

